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13. ABSTRACT (Maximum 200 Words) <p>Neurofibromatosis type 2 (NF2) is associated with a homozygous inactivation of the neurofibromatosis 2 gene (<i>NF2</i>). Despite intense study of the <i>NF2</i> gene, the mechanism by which the <i>NF2</i> tumor suppressor acts to prevent tumor formation is not well understood. The <i>NF2</i> transcripts undergo alternative splicing, generating a series of mRNA isoforms lacking one or more exons. Presently, the role of alternative splicing of <i>NF2</i> mRNAs is not understood. The <i>NF2</i> transcripts are also terminated at different polyadenylation sites. The role of this differential polyadenylation is not known. The goal of this research is to examine the role of posttranscriptional regulation (alternative splicing and differentiation polyadenylation) of the <i>NF2</i> gene. During this reporting period, we have completed the analysis of the pattern and relative frequency of alternatively spliced <i>NF2</i> isoforms expressed in vestibular schwannomas and various other human tissues and cells. We found that vestibular schwannomas express a distinct pattern of alternatively spliced <i>NF2</i> transcripts lacking specific exons. We have produced transgenic mice carrying a 2.4-kb <i>NF2</i> promoter-driven \square-gal construct have been produced. Analysis of transgene expression showed that the 2.4-kb <i>NF2</i> promoter could direct transgene expression to the nervous system during development. We also found that expression of the two schwannoma-expressed <i>NF2</i> isoforms, lacking exons 15 and 16, or exons 8 and 16, respectively, did not affect the growth properties of 293 and RT4 cells. We are presently producing transgenic mice carrying the schwannoma-expressed <i>NF2</i> cDNA isoforms driven by the human <i>NF2</i> promoter or the mouse P0 promoter to address whether these <i>NF2</i> isoforms possess any properties conducive to tumor formation <i>in vivo</i>.</p>			
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INTRODUCTION:

Neurofibromatosis type 2 (NF2) is associated with a homozygous inactivation of the neurofibromatosis 2 gene (*NF2*), which encodes a protein named 'merlin' for moesin-ezrin-radixin like protein (Trofatter et al., 1993). Despite intense study of the *NF2* tumor-suppressor, the mechanism by which merlin acts to prevent tumor formation is not well understood (reviewed in Gusella et al., 1999; Gutmann, 2001). The *NF2* transcripts undergo alternative splicing, generating a series of mRNA isoforms lacking one or more exons. Presently, the role of alternative splicing of *NF2* mRNAs is not understood. *NF2* isoform 1 (without exon 16) but not isoform 2 (containing all 17 exons) possess growth inhibitory properties (Gutmann et al., 1999). Also, transgenic mice over-expressing the *NF2* isoform with a deletion of exons 2 and 3 in Schwann cell lineage showed a high prevalence of Schwann cell hyperplasia and tumors (Giovannini et al., 1999). These results raise the possibility that functional contribution of the *Nf2* tumor suppressor may require a balanced expression of various isoform proteins in Schwann cells and/or other cell types. In addition, we found that differential usage of multiple polyadenylation sites also contributes to the complexity of human *NF2* transcripts (Chang et al., 2002). Presently, the role of differential polyadenylation of *NF2* transcripts is not known. The goal of the proposed research is to examine the role of posttranscriptional regulation (alternative splicing and differentiation polyadenylation) of the *NF2* gene. Ultimately we hope to provide a better understanding of the mechanisms of NF2 tumorigenesis.

BODY:**Aim 1: Analysis of the Expression Pattern of Alternatively Sliced *Nf2* Transcripts in Schwann Cells and Vestibular Schwannomas.**

Task 1: We have obtained our Institutional Review Board (IRB) approval for the three protocols and informed consents required for acquiring vestibular schwannomas and adjacent uninvolving vestibular nerves from NF2 patients, as well as normal human tissues. The institutional IRB approval letters together with the protocols were sent to Department of the Army Surgeon General's Human Subjects Research Review Board (HSRRB) at the Army Medical Research and Materiel Command. Final approval of the compliance has been obtained. Also, we have received approval from our IRB annual continuing review in July 2004.

Over the past year, 12 additional vestibular schwannomas and four paired normal vestibular nerves were procured. Written informed consent for tumors and nerves was obtained from all patients prior to surgical removal of their tumors. We have isolated RNAs from these schwannomas and vestibular nerves.

Task 2: To prepare Schwann cell cultures, we have learned the technique from Dr. Nancy Ratner's laboratory at the University of Cincinnati. We prepared primary cultures of Schwann cells from

freshly procured vestibular nerves accordingly (Ratner et al., 1986).

Task 3: Reverse transcription-polymerase chain reaction analysis were performed using RNAs from vestibular schwannomas, normal vestibular nerves, and various other normal human tissues including placenta, brain, heart, liver, and kidney. By cloning and sequencing analysis, we examined the complete exon structures and relative frequency of alternatively spliced *Nf2* isoforms (Chang et al., 2002). Our results showed that the expression pattern and relative frequency of the alternatively spliced *NF2* transcripts appeared to be different from those detected in other human tissues or cells. Particularly, in addition to isoforms I and II, these schwannomas expressed a high percentage of the *NF2* mRNA isoform lacking exons 15 and 16 (Neff et al., 2004. Laryngoscope, In Press).

In addition, we identified a sporadic schwannoma, which predominantly expressed *NF2* transcripts lacking exons 8, 15 and 16. Other cDNAs missing exon 8, exons 8 and 16, and exons 2, 8, 15, and 16 were also detected. Sequence comparison revealed that all of the cDNAs from this schwannoma completely matched the wild-type *NF2* sequence with the exception of the spliced exons.

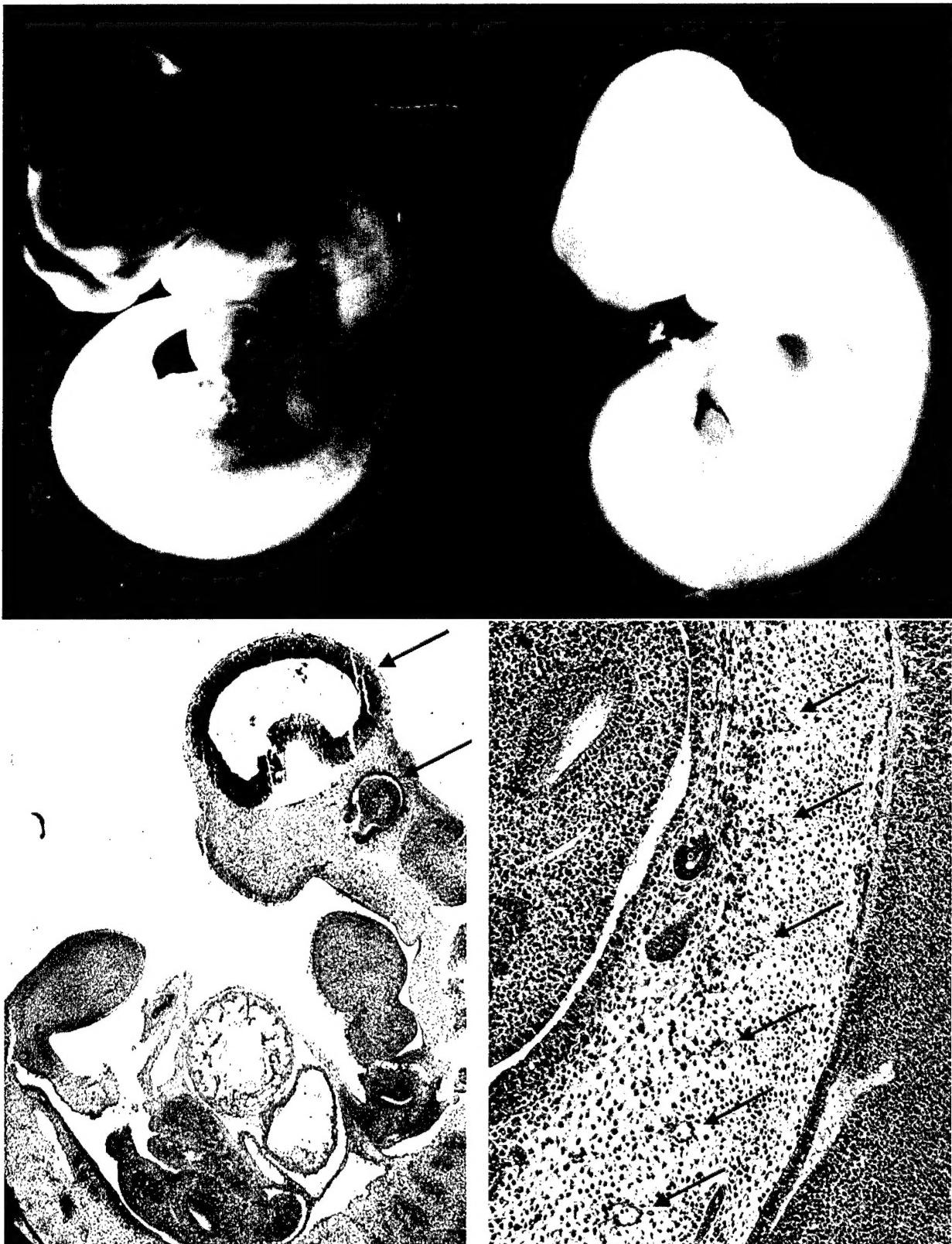
Taken together, these results indicate that vestibular schwannomas express a distinct pattern of alternatively spliced *NF2* transcripts lacking specific exons.

Task 4: We have isolated genomic DNA from the blood of the patient with a sporadic schwannoma that only expresses *NF2* mRNAs lacking at least exon 8.

Aim 2: Functional Analysis of the Two *NF2* Isoforms Commonly Expressed in Vestibular Schwannomas.

Task 5: The two *NF2* cDNA isoforms (lacking exons 15 and 16, or exon 8 and 16, respectively) commonly expressed in vestibular schwannomas have been cloned and placed under the control of a 2.4-kb human *NF2* promoter or a 1.1-kb mouse myelin P0 promoter.

Previously we showed that the 2.4-kb human *NF2* promoter could direct efficient expression of a reporter gene in transiently transfected cells (Chang et al., 2002). To examine whether the 2.4-kb *NF2* promoter is sufficient to direct expression to Schwann cells, we have produced five lines of transgenic mice carrying a 2.4-kb *NF2* promoter-driven β -galactosidase (β -gal) construct. To examine the expression pattern of transgene-encoded β -gal during embryonic development, we isolated transgenic embryos at various times post coitus (p.c.), and processed them for *X*-gal staining. As shown in the photographs below, β -gal expression was readily detected a transgenic embryo at 10.5 p.c., particularly in the head region (see below the photo on the top left), compared to a non-transgenic littermate. Analysis of sagittal tissue sections of the transgenic embryo revealed predominant β -gal expression in the developing neural tissues,



including those developing into the brain (indicated by arrows in the bottom left panel), and the cells surrounding the nerves in spine

(indicated by arrows in the bottom right panel). These results indicate that the 2.4-kb *NF2* promoter could direct transgene expression to the nervous system during development.

To examine the effect of over-expression of the two schwannoma-expressed *NF2* isoforms *in vivo*, we have generated transgenic constructs containing each corresponding *NF2* cDNA driven by the 2.4-kb *NF2* promoter or the 1.1-kb mouse P0 promoter. We are presently screening transgenic mice carrying these constructs.

Task 6: To test the biochemical and biological activities of the *NF2* cDNA isoforms *in vitro*, we have employed the pIND inducible expression system (Invitrogen). Hemagglutinin (HA) epitope-tagged *NF2* cDNA isoforms were constructed and inserted into the pIND vector. These inducible constructs carrying the HA-tagged *NF2* cDNA were co-transfected with the pVgRXR expression plasmid carrying a insect steroid hormone receptor into human adenovirus-transformed embryonic kidney 293 cells and rat RT4 schwannoma cells were performed.

Task 7: We first analyzed 293 cells co-transfected with an HA-tagged *NF2* cDNA expression vector and the pVgRXR plasmid. Upon induction with an insect steroid hormone ponasterone, expression of the HA-tagged *NF2* isoform protein was detected using an anti-HA antibody. However, little or no growth inhibitory or promoting effects on the 293 cells expressing either one of the two schwannoma-expressed *NF2* isoform proteins were seen.

We also performed a similar experiment using the rat RT-4 schwannoma cell line. RT4 were co-transfected with the *NF2* cDNA expression vector and pVgRXR. Upon hormone induction, expression of the schwannoma-expressed *NF2* isoforms also did not give rise to any effect on the growth properties of RT4 cells.

Task 8: To test protein-protein interaction, we fused the two schwannoma-expressed *NF2* isoforms with the glutathione-S-transferase (GST) protein. These GST-*NF2* fusion vectors have been used to produce fusion proteins. We are presently using these fusion proteins to examine the ability of the schwannoma-expressed *NF2* isoform proteins to form inter- and intra-molecular interaction.

Aim 3: Examination of the Potential Role of Differential Polyadenylation of *NF2* Transcripts.

Task 9: Expression plasmids for three human *NF2* cDNAs of 6.1, 3.1, and 2.7 kb in length, respectively, have been constructed. The 6.1-kb construct contains the full-length *NF2* cDNA with the longest 3' untranslated (UT) sequence. The 3.1- and 2.7-kb constructs have a shorter 3' UT region including different poly(A) signal sequences, as those found in the IMAGE2492737 and IMAGE324083 cDNAs (Chang et al., 2002). An HA epitope were also inserted to the N-terminus of the *NF2*-coding region to facilitate protein detection. To compare the RNA half-life expressed from these *NF* cDNA expression constructs, we are

presently transfecting these *NF2* cDNA expression plasmids into RT4 schwannoma cells, SK-N-AS neuroblastoma cells, and 293 kidney cells.

Task 10: We are also in the process of generating a series of 3' unidirectional deletion derivative of the 6.1-kb *NF2* expression plasmid. The end point of each deletion will be confirmed by sequencing.

Task 11: To test whether different 3' UT regions could affect the efficiency of protein translation, we are transfecting the 6.1-, 3.1- and 2.7-kb *NF2* cDNA expression plasmids carrying different lengths of the 3' UT sequences into RT4, SK-N-AS, and 293 cells. Western blot analysis are being performed to detect the level of *NF2* protein expression.

Task 12: During the past year, eight research abstracts were presented to national and local meetings. One research paper (Neff et al., 2004) will be published in the *Laryngoscope* and one manuscript (Lorenz et al., 2004) is presently under preparation.

KEY RESEARCH ACCOMPLISHMENTS:

(1) We have completed the analysis of the pattern and relative frequency of alternatively spliced *NF2* isoforms expressed in vestibular schwannomas and various other human tissues and cells. We found that vestibular schwannomas express a distinct pattern of alternatively spliced *NF2* transcripts lacking specific exons.

(2) Transgenic mice carrying a 2.4-kb *NF2* promoter-driven β -gal construct have been produced. Analysis of transgene expression showed that the 2.4-kb *NF2* promoter could direct transgene expression to the nervous system during development. Transgenic mice carrying the schwannoma-expressed *NF2* cDNA isoforms driven by the human *NF2* promoter or the mouse P0 promoter are being produced.

(3) Transfection study showed that over-expression of the two schwannoma-expressed *NF2* isoforms, lacking exons 15 and 16, or exons 8 and 16, respectively, did not affect growth properties of 293 and RT4 cells.

REPORTABLE OUTCOMES:

Eight research abstracts were presented to national and local meetings during the past year. Also, one research paper will be published in *The Laryngoscope* and one manuscript is in preparation.

Abstracts

(1) Yao, S. E.M. Akhmeteyeva, D.B. Welling, And L.-S. Chang. 2003. Expression of Novel Alternatively Spliced *NF2* cDNA isoforms in Vestibular Schwannomas. Eleventh Annual Department of Otolaryngology Alumni Symposium, Longaberger Alumni House, Columbus, OH.

We reported that vestibular schwannomas expressed novel alternatively spliced *NF2* RNA isoforms.

- (2) Chang, L.-S., E.M. Akhmametyeva, D.B. Welling, L. Lebedeva, N.A. Bulgakova, and L.V. Omelyanchuk. 2003. Expression and Function of the Neurofibromatosis 2 Gene During Early Development. Molecular Biology and Cancer Genetics Group Retreat, The Ohio State University Comprehensive Cancer Center, Deer Creek Park, OH.
- We showed that a 2.4-kb human NF2 promoter could direct a transgene expression to the developing neural tissues during early development. We also found that cells from the wing imaginal disc of the 3rd-instar fly larva lacking merlin showed abnormalities in the control of mitosis exit. Merlin mutant adult pharates have been isolated and displayed abnormal leg morphology. In addition, the wingless protein expression in the dorsal/ventral compartment border was not found in the merlin mutant wing imaginal disc.*
- (3) Akhmametyeva, E.M., L. Lebedeva, S.A. Trunova, N.A. Bulgakova, L.V. Omelyanchuk, and L.-S. Chang. 2004. Developmental Expression of the NF2 Gene and Genetic Analysis of Merlin Function. Columbus Children's Research Institute Annual Research Conference, Columbus, OH.
- We analyzed the developmental expression pattern of the human NF2 gene promoter in transgenic mice and conducted further genetic analysis to identify merlin function during fly development.*
- (4) Akhmametyeva, E.M., L. Lebedeva, S.A. Trunova, N.A. Bulgakova, L.V. Omelyanchuk, and Long-Sheng Chang. 2004. Developmental Expression of the NF2 Gene and Genetic Analysis of Merlin Function. The 6th Annual Comprehensive Cancer Center Scientific Meeting, Columbus, OH.
- We reported the developmental expression pattern of a 2.4-kb human NF2 promoter in transgenic mice. We also showed that merlin is important for the control of mitosis exit. Our results further suggest that merlin plays an important role in the determination and/or maintenance of the dorsal/ventral compartment border in the wing imaginal disc.*
- (5) Akhmametyeva, E.M., D.B. Welling, and Chang, L.-S. 2004. Expression of the Neurofibromatosis 2 Gene During Early Development. Pediatric Academic Societies' Meeting, San Francisco, CA.
- We reported that the early embryonic expression pattern of the human NF2 gene promoter in transgenic mice.*
- (6) Welling, D.B., BA. Neff, Brenda Hall, John M. Lasak, and L.-S. Chang. 2004. Long-Term Hearing Rehabilitation in NF2 Patients With Cochlear Implants. The NNFF International Consortium for Molecular Biology of NF1 And NF2, Aspen, CO.
- We presented long-term follow-up on 6 patients who received cochlear implantation following vestibular schwannoma removal. All patients continued to show very*

good to excellent speech understanding. These results suggest that early intervention with preservation of the anatomic integrity of the cochlear nerve appears warranted even if hearing preservation is not accomplished at the time of tumor removal. Early detection and treatment of NF2 provides much greater opportunity for hearing preservation

- (7) Lorenz, M., B.A. Neff, E.M. Akhmeteva, J. Rock, J. Shoreman, S. Tae, P. Schmalbrock, D.B. Welling, and L.-S. Chang. 2004. Vestibular Schwannomas Xenografted in SCID Mice and Expression of Cyclin D1, D3, and Telomerase. The NNFF International Consortium for Molecular Biology of NF1 And NF2, Aspen, CO. We examined and compared the growth of human vestibular schwannomas and two malignant rat schwannoma cell lines transplanted in SCID mice by histology and magnetic resonance imaging. We also reported that five of the ten human vestibular schwannomas examined showed moderate staining of cyclin D3, while none of the 15 tumors examined showed cyclin D1 expression. In addition, we found that vestibular schwannomas contained moderate-sized telomeres. In contrast, malignant schwannomas harbored long telomere sequences. Taken together, our results suggest multiple factors contribute to the growth potential of human vestibular schwannomas
- (8) Chang, L.-S., E.M. Akhmeteva, L. Lebedeva, N.A. Bulgakova, and L.V. Omelyanchuk. 2004. Developmental Expression of the NF2 Gene and Genetic Analysis of Merlin Function. The NNFF International Consortium for Molecular Biology of NF1 And NF2, Aspen, CO.
- To understand NF2 expression during development, we produced transgenic mice carrying a 2.4-kb NF2 promoter-driven LacZ gene. The NF2 promoter could direct transgene expression during early development. Analysis of transgenic embryos At 10.5 p.c. showed that intense transgene-encoded β -galactosidase expression was detected mostly in the developing neural tissues, including those developing into the brain and nervous system. To better understand merlin function during development, we analyze merlin mutant flies and found that the Mer⁴ mutant cells (residue 170^{Gln-stop}) from the wing imaginal disc exhibited asynchronous anaphase-telophase transition – two sister chromosome sets showed different degrees of chromosome decondensation. We also isolated adult hemizygous Mer⁴ males which displayed abnormal leg morphology, with bowed femurs and tibias. Duplication of the wing disc in Mer⁴ hemizygous larvae was also seen. In addition, we found that wingless protein expression at the dorsal/ventral compartment border was not detected in the wing disc of Mer⁴ larvae. These results indicate that merlin plays important roles in mitosis exit control and development.

Publication and Manuscript

- (1) Neff, B.A., D.B. Welling, E. Akhmeteva, and L.-S. Chang. 2004. The Molecular Biology of Vestibular Schwannomas: Dissecting the Pathogenic Process at the Molecular Level. Laryngoscope, In Press.
- In this article, we review the clinical characteristics of vestibular

schwannomas and neurofibromatosis type 2 (NF2) syndromes with relation to alterations in the NF2 gene, including mutations and alternative splicing. We also discuss merlin, the protein product of the NF2 gene and how merlin interacts with other proteins that may lead to a better understanding of NF2 function. By using recently developed cDNA microarray technology, genes or pathways that are deregulated in vestibular schwannomas have been identified. Avenues for the development of potential future therapies are highlighted

- (2) Lorenz, M.B., B.A. Neff, E.M. Akhmadetyeva, J. Rock, E.P. Oberstein, J. Shoreman, S. Tae, P. Schmalbrock, A.R. Chaudhury, J. Yamate, E. Dodson, D.B. Welling, and L.-S. Chang. 2004. Vestibular Schwannomas Xenografted in Scid Mice and Expression of Cyclin D1, D3, and Telomerase. Manuscript In Preparation. This article describes the comparison of the growth of human vestibular schwannomas and two malignant rat schwannoma cell lines transplanted in SCID mice by histology and magnetic resonance imaging. Analysis of potential factors including cyclin D1 and D3 expression and the length of telomere that could affect schwannoma cell growth are also reported. Our results suggest multiple factors contribute to the growth potential of human vestibular schwannomas

CONCLUSIONS:

Vestibular schwannomas express a distinct pattern of alternatively spliced NF2 transcripts lacking specific exons, suggesting that these alternatively spliced exons may be important for NF2 function. Further experiments are in progress to address whether these alternative splicing NF2 isoforms preferentially expressed in schwannomas possess any additional properties conducive to tumor formation *in vivo*.

REFERENCES:

- Chang, L.-S., Akhmadetyeva, E.M., Wu, Y., and Welling, D.B. 2002. Multiple transcription initiation sites, alternative splicing, and differential polyadenylation contribute to the complexity of human neurofibromatosis 2 transcripts. *Genomics* 79:63-76.
- Giovannini M, Robanus-Maandag E, Niwa-Kawakita M, van der Valk M, Woodruff JM, Goutebroze L, Merel P, Berns A, Thomas G. 1999. Schwann cell hyperplasia and tumors in transgenic mice expressing a naturally occurring mutant NF2 protein. *Genes Dev.* 13:978-986.
- Gusella JF, Ramesh V, MacCollin M, Jacoby LB. 1999. Merlin: the neurofibromatosis 2 tumor suppressor. *Biochim. Biophys. Acta* 1423:M29-M36.
- Gutmann, D.H. 2001. The neurofibromatoses: when less is more. *Hum. Mol. Genet.* 10:747-55.
- Gutmann DH, Sherman L, Seftor L, Haipek C, Hoang Lu K, Hendrix M. 1999. Increased expression of the NF2 tumor suppressor gene product, merlin, impairs cell motility, adhesion and spreading. *Hum. Mol. Genet.* 8:267-275.
- Neff, B.A., D.B. Welling, E. Akhmadetyeva, and L.-S. Chang. 2004. The

molecular biology of vestibular schwannomas: Dissecting the pathogenic process at the molecular level. Laryngoscope, In Press.
Ratner, N., Bunge, R.P., Glaser, L. 1986. Schwann cell proliferation in vitro. An overview. Ann N Y Acad Sci.486:170-181
Trofatter, J.A., MacCollin, M.M., Rutter, J.L., Murrell, J.R., Duyao, M.P., Parry, D.M., Eldridge, R., Kley, N., Menon, A.G., Pulaski, K., Haase, V.H., Ambrose, C.M., Munroe, D., Bove, C., Haines, J.L., Martuza, R.L., MacDonald, M.E., Seizinger, B.R., Short, M.P., Buckler, A.L., Gusella, J.F. 1993. A novel Moesin-, Exrin-, Radixin-like gene is a candidate for the neurofibromatosis 2 tumor-suppressor. Cell 72:791-800

APPENDICES:

Research

Publication and Manuscript

- (1) Neff, B.A., D.B. Welling, E. Akhmettyeva, and L.-S. Chang. 2004. The Molecular Biology of Vestibular Schwannomas: Dissecting the Pathogenic Process at the Molecular Level. Laryngoscope, In Press.
- (2) Lorenz, M.B., B.A. Neff, E.M. Akhmettyeva, J. Rock, E.P. Oberstein, J. Shoreman, S. Tae, P. Schmalbrock, A.R. Chaudhury, J. Yamate, E. Dodson, D.B. Welling, and L.-S. Chang. 2004. Vestibular Schwannomas Xenografted in Scid Mice and Expression of Cyclin D1, D3, and Telomerase. Manuscript In Preparation.

**The Molecular Biology of Vestibular Schwannomas:
Dissecting the Pathogenic Process at the Molecular Level**

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ABSTRACT

Recent advances in molecular biology have led to a better understanding of the etiology of vestibular schwannomas. Mutations in the neurofibromatosis type 2 tumor suppressor gene (*NF2*) have been identified in vestibular schwannomas. The clinical characteristics of vestibular schwannomas and neurofibromatosis type 2 (*NF2*) syndromes will be reviewed and related to alterations in the *NF2* gene. Additionally, merlin, the protein product of the *NF2* gene, will be discussed. The discovery of how merlin interacts with other proteins may lead to a better understanding of *NF2* function. By using recently developed cDNA microarray technology, genes or pathways that are deregulated in vestibular schwannomas have been identified. Avenues for the development of potential future therapies will be highlighted.

Introduction

Vestibular schwannomas are histologically benign tumors of the neural sheath that originate on the superior or inferior vestibular branches of cranial nerve VIII. The term “vestibular schwannoma” is preferred over the more commonly used term “acoustic neuroma” because these tumors are neither neuromas, nor do they arise from the acoustic (cochlear) nerve. They occur either as sporadic unilateral tumors or bilateral tumors; the development of bilateral vestibular schwannomas is the hallmark of neurofibromatosis type 2 (NF2). Various types of vestibular schwannomas can be loosely grouped into unilateral sporadic vestibular schwannomas, bilateral or NF2-associated schwannomas, and cystic schwannomas.

Unilateral schwannomas are the most common presentation, and they constitute 95% of all vestibular schwannomas. Sporadic vestibular schwannomas occur in approximately 10 persons per million per year (1,2). However, the true incidence may be higher, as highlighted by Anderson et al., who demonstrated an incidence of 7 unsuspected schwannomas per 10,000 brain MRI studies (0.07%) (3). Sporadic tumors usually occur in the 4th and 5th decades with a mean presentation of 50 years of age. Although histologically benign, schwannomas can, if large enough, cause hydrocephalus, brain stem compression, herniation, and death. Most commonly, however, they are associated with hearing loss, tinnitus, imbalance, and other symptoms related to compression of adjacent cranial nerves.

NF2 is clinically an autosomal dominant disease that is highly penetrant (4). NF2-associated tumors account for about 5% of all vestibular schwannomas. Patients who inherit an abnormal copy of the NF2 tumor suppressor gene have a 95% chance of developing bilateral vestibular schwannomas. However, about one half of the cases have no family history of NF2, and thus, they represent new germline mutations that were not inherited. Other disease features of NF2 include intracranial meningiomas, ependymomas, spinal schwannomas, and presenile lens opacities (5-8). NF2 is now recognized as a disease that is distinctly different from neurofibromatosis type 1 (NF1) or von Recklinghausen’s disease. NF1, which is associated with multiple peripheral neuromas, is caused by a mutation in the NF1 tumor suppressor gene on chromosome 17.

NF2 is currently subdivided into three groups (9,10). The *Wishart* type has a more severe clinical

presentation. In addition to bilateral vestibular schwannomas, patients often suffer from associated spinal tumors with a typical onset in the late teens or early twenties (11). The *Gardner* type has a later onset and a less severe presentation. Although patients present with bilateral schwannomas, the incidence of associated intracranial tumors is less common. (12). A more recently recognized third category of NF2 has been termed *segmental* NF2. The cause of segmental NF2 may be due to somatic mosaicism where a mutation occurs in embryogenesis rather than in the germline DNA; therefore, only a portion of the patients' cells carries the mutation (13,14). This is different from those patients with traditional NF2 who inherit the mutation from their parent. Kluwe et al. recently estimated that mosaicism may account for 24.8% (58/233) of the NF2 cases of any subtype among patients whose parents did not display the disease (14). Patients with somatic mosaicism can display bilateral vestibular schwannomas if the postzygotic mutation occurred early in embryogenesis. However, they may also display an atypical presentation, or *segmental* NF2, in which the patient has a unilateral vestibular schwannoma and an ipsilateral, additional intracranial tumor, such as a meningioma, if the postzygotic mutation occurred late in development (13). Unlike the traditional forms of NF2, the risk of passing NF2 caused by mosaicism to future offspring is very low (15).

Schwannomatosis, a recently defined form of neurofibromatosis, is characterized by multiple schwannomas without any vestibular schwannomas, diagnostic of NF2. Patients with schwannomatosis frequently present with intractable pain rather than cranial nerve deficits. They do not develop other intracranial tumors or malignancies. MacCollin et al. noted that about one third of patients with schwannomatosis had tumors in an anatomically limited distribution, such as a single limb, several contiguous segments of spine, or one half of the body (16). Sporadic cases of schwannomatosis are as common as NF2, but few cases of familial schwannomatosis have been identified. This is in contradistinction to NF1 and NF2, which are autosomal dominant, highly penetrant syndromes that are frequently found clustered in families. The underlying molecular disruption in schwannomatosis is a pattern of somatic *NF2 gene* inactivation incompatible with NF1 or NF2, but this has not been completely defined.

Cystic vestibular schwannomas are a particularly aggressive group of unilateral, sporadic schwannomas,

which invade the surrounding cranial nerves, splaying them throughout the tumors. Cystic vestibular schwannomas are associated with either intratumoral or extratumoral cysts which develop in the loosely organized Antoni B tissues. In addition, a higher degree of nuclear atypia is seen in cystic tumors (17,18). Careful distinction must be drawn between the truly cystic schwannomas and the very common heterogeneous schwannomas which are not as aggressive in their clinical behavior. Magnetic resonance imaging (MRI) distinguishes clearly between the solid and cystic vestibular schwannomas. Cystic regions of the tumors are hyper-intense on T2-weighted images, and the cysts do not enhance with gadolinium administration. The non-cystic component of the cystic tumors enhances with gadolinium in a manner similar to the unilateral and NF2-associated schwannomas (**figure I**). Cystic tumors may grow rapidly, and they are very difficult to manage due to the high rate of hearing loss and facial nerve paralysis that occurs after surgical removal (19). When compared to solid tumors of a similar size, the rate of complete facial nerve paralysis (House-Brachmann grade VI) with surgical removal of cystic tumors was 41%, as compared to 27 % for that of solid unilateral schwannomas (20). Cystic tumors are also more likely to have continued growth and facial nerve paralysis even with stereotactic radiation treatments than either the unilateral spontaneous or NF2- associated schwannomas (21,22).

Although the effectiveness of treatment with current surgical and radiation treatments for vestibular schwannomas are generally good, treatment-related morbidity continues to be problematic. The field of molecular biology is proposed as the discipline to advance the level of diagnosis and to improve the treatment of vestibular schwannomas. When applied to various neurologic pathologies, “molecular neurotology” may soon develop as a medical discipline in a manner similar to the advent of surgical neurotology in the 1960’s. A brief review of the recent discoveries and advances in the molecular biology of vestibular schwannomas will follow.

The *NF2* gene

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this has not held true in other studies, which showed that some missense mutations associated with a severe phenotype. In addition, missense mutations within the α -helical domain of the *NF2* protein appear to associate with a less severe phenotype than those within the conserved ERM domain (58). This lack of genotype-phenotype correlation was also seen for large deletions which could give rise to mild phenotypes as well as the previously reported severe disease expression (59). Given the heterogeneity of clinical response to various types of mutations, no clear genotype to phenotype correlation has been established, and this is further evidenced by the fact that phenotypic variability within the *NF2* families with the same mutation has been seen (60). By extensive screening of the *NF2* gene, Zucman-Rocci reported an 88% mutation detection rate in vestibular schwannomas; thus, additional mechanisms for inactivation of the *NF2* gene in some patients may exist (61). The possibility of a modifier gene has been suggested (62). Also, mutation or methylation in the regulatory region of the *NF2* gene has been suggested as a possible mechanism of gene inactivation (63,64). The complexity of *NF2* transcripts generated by post-transcriptional alternative splicing and differential polyadenylation may also be considered as possible means of inactivating the *NF2* gene (64).

The NF2 protein- structure and function

The *NF2*-coding region encompasses 17 exons spanning 90 kilo base-pairs of DNA on chromosome 22 (25,26,61). It encodes a 595-amino acid protein product which has been named *merlin* (for moesin-ezrin-radixin like protein) or *schwannomin* (derived from schwannoma) (25,26). For simplicity, the *NF2* protein will be referred to as “merlin” in this manuscript.

Merlin shares a high degree of homology to the erythrocyte protein 4.1-related superfamily of proteins, which act to link the actin cytoskeleton to the plasma membrane. In particular, three proteins, ezrin, radixin, and moesin, referred to as the ERM family, share a great deal of structural similarity with merlin (26,65). The proteins belonging to this family all have a similar N-terminal globular domain, also known as the FERM domain, followed by an α -helical stretch, and finally a charged carboxyl-terminus (66). The key functional domains of merlin may lie within the highly conserved FERM domains and the unique C-terminus of the

protein. The ERM proteins have been shown to be involved in cellular remodeling involving the actin cytoskeleton (67). These proteins bind actin filaments in the cytoskeleton via a conserved C-terminal domain and possibly via a second actin-binding site in the N-terminal half of the protein (68,69).

Like the ERM proteins, merlin is expressed in a variety of cell types where it localizes to the areas of membrane remodeling, particularly membrane ruffles, although its precise distribution may differ from the ERM proteins expressed in the same cell (70). Interestingly, schwannoma cells from NF2 tumors show dramatic alterations in the actin cytoskeleton and display abnormalities in cell spreading (71). These results suggest that merlin may play an important role in regulating both the actin cytoskeleton-mediated processes and cell proliferation (72). However, it should be noted that merlin has a growth suppression role, while other ERM-family members seem to facilitate cell growth.

Merlin acts as a tumor suppressor

Over-expression of the *nf2* gene in mouse fibroblasts or rat schwannoma cells can limit cell growth (56,73,74) and suppress cell transformation by the ras oncogene (75). The growth control of certain Schwann cells and meningeal cells is lost in the absence of *NF2* function, suggesting that *NF2* mutations and merlin deficiency disrupt some aspect of intracellular signaling that leads to cellular transformation. These findings demonstrate merlin's ability to act as a tumor suppressor.

Furthermore, scientists have developed *nf2* knockout mice which were designed to be missing one or both copies of the *nf2* gene in the germline. Intriguingly, heterozygous *nf2* knockout mice go on to develop osteosarcomas, and less often, fibrosarcomas or hepatocellular carcinomas (76). Genetic analysis of these tumors shows that nearly all of them are missing both *nf2* alleles due to a mutation causing a loss of the second *nf2* allele. The fact that tumor growth occurs in the absence of both *nf2* alleles indicates that the *nf2* gene possesses a classical tumor suppressor function. However, none of the heterozygous *nf2* mice develop tumors or clinical manifestations associated with human NF2.

Homozygous *nf2* mutant mice, which are designed to be missing both *nf2* alleles also do not demonstrate

clinical characteristics of human NF2, and the mutant embryos die at approximately seven days of gestation, indicating that a homozygous *nf2* mutation is embryonic lethal (77). Together with our preliminary data showing that the *nf2* gene is expressed early in embryogenesis (our unpublished data), these results indicate that the *nf2* gene product plays an important role during early embryonic development.

By engineering mice whose Schwann cells have exon 2 excised and removed from both *nf2* alleles, conditional homozygous *nf2* knockout mice have been produced which display some characteristics of NF2 including schwannomas, schwann cell hyperplasia, cataracts, and osseous metaplasia (78). Although these results argue that loss of merlin is sufficient for schwannoma formation *in vivo*, none of the tumors observed in these conditional knockout mice were found on the vestibular nerve. This is in contrast to those vestibular schwannomas commonly found in patients with NF2. Further work is needed to develop a mouse model that phenotypically displays schwannomas originating from the eighth cranial nerve.

Merlin cell signaling and regulation

In addition to the actin cytoskeleton, merlin has been shown to associate with cell membrane domains, which are highly enriched in signaling molecules that regulate cellular responses to proliferative and antiproliferative stimuli (79). To date, several proteins that are likely to interact with merlin have been identified. These include the ERM proteins, hyalurin receptor CD44, F-actin, paxillin, microtubules, β II-spectrin, β 1-integrin, β -fodrin, the regulatory cofactor of $\text{Na}^+ - \text{H}^+$ exchanger (NHE-RF), SCHIP-1, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), p21-activated kinase 1 and 2 (Pak1 and Pak2), Rac1, and RIB subunit of protein kinase A (74,80-94).

Presently, how these protein-protein interactions relate to the tumor suppressor activity of merlin is largely not understood. The association of merlin with CD44 and β 1-integrin raises the possibility that merlin might function as a molecular switch in the signaling pathways. CD44 is a transmembrane hyaluronic acid receptor implicated in cell-cell adhesion, cell-matrix adhesion, cell motility, and metastasis (93,94). Recent evidence suggests that merlin mediates contact inhibition of cell growth through signals from the extracellular matrix. At

high cell density, merlin becomes hypo-phosphorylated and inhibits cell growth in response to hyaluronate, a mucopolysaccharide that surrounds cells. Merlin's growth-inhibitory activity depends on specific interaction with the cytoplasmic tail of CD44. At low cell density, merlin is phosphorylated, growth permissive, and exists in a complex with ezrin, moesin, and CD44. These data indicate that merlin and CD44 form a molecular switch that specifies cell growth arrest or proliferation (95). Rac1, a member of the RhoGTPase family, has recently been demonstrated to promote phosphorylation of merlin thereby inactivating its growth suppressor mechanism. Additionally, among the Rac/Cdc42 effectors, p21-activated kinase 2 (Pak2) has been shown to phosphorylate merlin at serine 518 and inactivates its function (79,96,97). Kissil et al. also recently reported an interaction between merlin and Pak1. Merlin inhibits the activation dynamic of Pak1. Loss of merlin expression leads to the inappropriate activation of Pak1, while overexpression of merlin results in the inhibition of Pak1 activity (82). (**Figure II**)

Merlin's growth regulatory function is related to its conformation and protein-protein interactions

The activities of the ERM proteins are controlled by self-association of the proteins' N-terminal and C-terminal regions (98,99). The ERM proteins can exist in the 'closed' conformation, where the N- and C-terminal regions undergo an intramolecular interaction, thus folding the protein to mask the conserved actin-binding site (Figure III). The molecule can be converted to the 'open' conformation in which the intramolecular interaction is disrupted by signals such as phosphorylation or treatment with phosphoinositides (56,82,97,100).

Merlin's ability to function as a growth regulator is also related to its ability to form such intramolecular associations. Two such interactions have been identified. The first interaction is between residues which fold the N-terminal end of the protein onto itself, while the second interaction folds the entire protein so that there is contact between N and C-terminal ends of the protein (56,74,101,102) (**Figure II**). In a fashion similar to the ERM proteins, merlin may cycle between the 'open' and 'closed' conformations that differentially determine whether it binds with the ERM proteins or other molecules to transduce merlin's growth inhibition signal (103). In addition, the association between merlin and HRS, a substrate implicated in the signaling pathway initiated

by hepatocyte growth factor (HGF) binding to the c-met receptor (104), appears to be regulated by merlin folding, suggesting that the ability of merlin to cycle between the ‘open’ and ‘closed’ conformations may integrate CD44 and HGF signaling pathways relevant to growth regulation (102).

The *NF2* gene promoter

Another research focus, which is currently being investigated, is to characterize the upstream and downstream untranslated regions of the *NF2* gene so that these regions could be screened for mutations in both spontaneous and familial tumors in which no mutation was found in the *NF2*-coding region. We have mapped the major transcription initiation site of the *NF2* gene and found that multiple regions in the *NF2* promoter are required for full *NF2* promoter activity (64,105). Both positive and negative *cis*-acting regulatory elements required for transcription of the *NF2* gene have been found in the 5' flanking region of the promoter. A G/C-rich sequence located in the proximal promoter region, which can be bound by the Sp1 transcription factor, serves as a positive regulatory element. Both the 5' and 3' flanking regions of the human *NF2* locus are G/C rich and could serve as a target for gene methylation and inactivation (64).

Alternatively spliced *NF2* mRNA isoforms in vestibular schwannomas and other cell types

DNA consists of regions called exons and introns. The exons are the segments of DNA that are transcribed and brought together as a mature mRNA product. The introns represent the sections of DNA that are transcribed but are spliced out during RNA processing. Alternate splicing is the mechanism by which different exon combinations are brought together to produce multiple mRNA transcripts from the same gene. These alternately spliced transcripts can include all of the gene’s exons, or can be missing one or multiple exons. The different RNA transcripts produced from this process are termed mRNA isoforms.

An example of *NF2* mRNA isoforms is shown in **Figure III**. The *NF2* gene undergoes alternative splicing in the coding exons. Multiple alternatively spliced *NF2* transcripts have been identified in various human cells. The most common isoforms in these cells were isoforms II (containing all 17 exons) and I (without exon 16)

(33,64,106-111)

We have also examined the expression of alternatively spliced *NF2* mRNA isoforms in vestibular schwannomas (1 *NF2* schwannoma, 7 sporadic schwannomas, and 2 cystic schwannomas). Cloning and sequencing analysis showed that the expression pattern and relative frequency of the alternatively spliced *NF2* transcripts appeared to be different from those detected in other human cell types described above. Particularly, in addition to isoforms I and II, these schwannomas expressed a high percentage of the *NF2* mRNA isoform lacking exons 15 and 16. These alternatively spliced *NF2* transcripts could encode different protein products. (our unpublished data)

Presently, the role of alternative splicing of *NF2* mRNA is not well understood. It is possible that the functional contribution of the *NF2* tumor suppressor may require a balanced expression of various isoform proteins in Schwann cells and/or other cell lineages (64,112). Alternatively, alternative splicing may be another mechanism for Schwann cells to inactivate merlin function and/or to generate isoforms that have additional properties conducive to tumor formation. We are presently conducting experiments to test these possibilities.

Immunohistochemical markers of growth in vestibular schwannomas: clinical correlation

Attempts to correlate clinical parameters with immunohistologic evaluation of protein expression in vestibular schwannomas have been performed. An increase in Ki-67, which is an index of nuclear proliferation, was shown to correlate with the growth of solid schwannomas on MRI (113, 114). Higher rates of tumor recurrence have also been suggested in tumors with an increased rate of nuclear proliferation and mitotic indexes, although the supporting data for this claim is not conclusive (115). Positron emission tomography (PET) scanning has been conducted to assess the metabolic activity of vestibular schwannomas preoperatively and to correlate the metabolic activity with the proliferation index, Ki-67. No correlation was found between the large and recurrent tumors and the uptake of 18-fluorodeoxyglucose (FDG) as a radionuclide tracer to measure glucose metabolism by PET scanning. Additionally, there was no correlation between FDG uptake and Ki-67 expression measured by immunostaining (116). A possible reason for this is that vestibular

schwannomas are slow growing tumors with only a small proportion of the tumor cells being in S-phase (active division) (117).

Another possible marker for tumor growth is the transforming growth factor β 1 (TGF- β 1). Immunostaining for TGF- β 1 was positive in 96% of blood vessels within schwannomas and in 84% of schwannoma tissue samples; however again, no clinical correlation with tumor types or tumor growth was found (118). Immunohistochemical association of β 1-integrin with merlin has been demonstrated, but has not been related directly to tumor phenotypes (88). Cystic schwannomas are associated with a 36-fold decrease in nuclear proliferation as measured with Ki-67 staining when compared to solid tumors, suggesting that the rapid clinical growth seen in cystic schwannomas is related to the accumulation of cyst formation but not by an actual increase in the growth rate of tumor cells (119,120). Also, NF2-associated schwannomas have been shown to have an increased proliferation index by Ki-67 and PCNA immunostaining, when compared to unilateral solid schwannomas (121,122).

Taken together, these studies demonstrate a degree of correlation between clinical growth as assessed by MRI scans and historical data, and nuclear growth indexes in solid unilateral and *NF2*-associated schwannomas. However, cystic tumor growth appears to occur via a different mechanism. Although the defective *NF2* gene is the underlying common denominator in tumor formation of unilateral sporadic, *NF2*-associated, and cystic schwannomas, other differences at the molecular level likely account for the variable clinical presentations of these tumors.

Identifying deregulated genes in vestibular schwannomas

With 69,227 mRNA sequences representing unique human genes and more than 3 million expressed sequence tags (ESTs) in the UniGene data base, the success of the Human Genome Project is evident. However, the expression, function, and regulation of the majority of genes are not yet known (123). The study of large-scale gene expression profiles utilizing cDNA microarrays allows examination of the so-called ‘transcriptome’ of a tissue, and gives a means of exploring a broad view of the basic biology of tumors

(124,125). Data from the human genome project makes the expression profiles more readily searchable, and organization of the genes into functional groupings allows examination of distinct pathways. For example, cell cycle control, DNA damage repair, or signal transduction and transcription factors can be organized and reviewed for various tumors (126). Biochips that contain thousands of oligonucleotides representing genes from the human genome have been created and are used to perform cDNA microarrays.

To evaluate the gene expression profile in a tumor, RNA is isolated from the tumor and converted into cDNA or cRNA. This cDNA or cRNA is then labeled with a fluorescent dye and hybridized to the oligonucleotides on the biochip. The same process can be used to evaluate RNA expressed in a normal tissue and compare gene expression differences between the diseased and normal tissues. Consequently, this helps scientists identify deregulated genes in the diseased tissue. Microarray gene expression analysis has been successfully utilized in recent years to evaluate a number of solid tumors including breast carcinoma, colon carcinoma, prostate carcinoma, ovarian carcinoma, and vestibular schwannomas (127-134).

Gene expression analysis has revealed differences among tumors which are not distinguishable histologically. Molecular classification, rather than histological classification, may also better predict the response of certain tumor types to specific therapies (135). This genomic scale approach has helped to identify sub-classes of colon carcinoma, breast carcinoma, melanoma, leukemias and lymphomas (132-134). In several instances, cDNA microarray analysis has already identified genes that appear to be useful for predicting clinical behavior.

Vestibular schwannoma characteristics can not be explained by the current understanding of the mutation types alone. Investigating inter-tumor variability of gene expression profiles shows promise to help unravel the clinical differences among subtypes of vestibular schwannomas. To better understand the pathways leading to schwannoma formation, we have utilized cDNA microarray analysis to evaluate gene expression profiles of vestibular schwannomas (127,128). Three sporadic vestibular schwannomas, two NF2-associated schwannomas, and three cystic schwannomas were compared to a normal vestibular nerve from a patient with a sporadic schwannoma. The goal was to seek patterns of gene expression consistently elevated or decreased

across all tumors. Of 25,920 genes or ESTs screened, 42 genes were significantly up-regulated (by a factor of three or more) consistently across at least 6 of the 8 tumors examined. Additionally, multiple genes were found to be significantly down-regulated in the majority of vestibular schwannomas examined. Of these genes, eight genes involved with cell signaling and division were down-regulated, including an apoptosis-related, putative tumor suppressor gene LUCA-15 which was down-regulated in 7 of 8 schwannomas studied.

Interesting and potentially important pathways for tumorigenesis are suggested by the deregulated genes. Two mediators of angiogenesis, endoglin and osteonectin, were highly elevated in most, if not all, tumors examined (127). Endoglin is a transforming growth factor- α receptor that is known to be an endothelial marker for angiogenesis in solid tumors, and osteonectin is a secreted glycoprotein that interacts with extracellular matrix proteins to decrease adhesion of cells from the matrix, thereby inducing a biological state conducive to cell migration. Endoglin was found to be significantly up-regulated in all of the solid tumors but not in any of the cystic tumors examined. The difference in endoglin gene expression may be a key to unlocking why some schwannomas develop into the aggressive cystic phenotype. Osteonectin was elevated in all of the tumors studied and may be a target for potential therapies including angiogenesis inhibitors (127,128,136,137). An example of a deregulated signaling pathway suggested by the microarray data is the retinoblastoma protein (pRb)-cyclin-dependent kinase (CDK) pathway (128). Among genes involved in G₁-S progression, CDK2 was found to be down-regulated in 7 of 8 tumors. In addition, up-regulation of transforming factor RhoB was found in all of the schwannomas examined (127)

Summary

The discovery of molecular mechanisms underlying vestibular schwannoma formation is rapidly moving forward. Understanding merlin's interactions with other proteins, signaling pathways, and regulation of the *NF2* gene will hopefully lead to the development of novel treatments for vestibular schwannomas. These treatments may not be as far in the future as one might think. For example, upregulation of the RhoB GTPase seen in vestibular schwannomas may respond to target specific therapy with Rho B inhibitors (138).

Ultimately, drug therapies will be designed to stop schwannoma progression. This will offer alternatives to the current options of untreated observation of tumor growth, stereotactic radiation, or surgical removal. Furthermore, it may also be possible to develop targeted therapy that may shrink or altogether eradicate preexisting tumors. These are the challenges facing the “molecular neurologist” of the future.

REFERENCES

1. Tos M, Thomsen J, Charabi S. Incidence of acoustic neuromas. *Ear Nose Throat J.* 1992; 71(9): 391-3.
2. Howitz MF, Johansen C, Tos M, et al. Incidence of vestibular schwannoma in Denmark, 1977-1995. *Am J Otol.* 2000; 21(5): 690-4.
3. Anderson TD, Loevner LA, Bigelow DC, et al. Prevalence of unsuspected acoustic neuroma found by magnetic resonance imaging. *Otolaryngol Head Neck Surg.* 2000; 122(5); 643-6.
4. Bull. World Health Org. Prevention and control of neurofibromatosis: Memorandum from a joint WHO/NNFF meeting. 1992; 70: 173-182.
5. Fontaine B, Rouleau GA, Seizinger BR, et al. Molecular genetics of neurofibromatosis 2 and related tumors (Acoustic neuroma and meningioma). *Ann NY Acad Sci.* 1991; 615: 338-343.
6. Kaiser-Kupfer M, Freidlin V, Datiles MB, et al. The association of posterior capsular lens opacities with bilateral acoustic neuromas in patients with neurofibromatosis type 2. *Arch Ophthalmol.* 1989; 107(4):541-544.
7. Kanter WR, Eldridge R, Fabricant R, et al. Central neurofibromatosis with bilateral acoustic neuroma: Genetic, clinical and biochemical distinctions from peripheral neurofibromatosis. *Neurology.* 1980; 30:851-859.
8. Martuza RL, Eldridge R. Neurofibromatosis 2 (Bilateral acoustic neurofibromatosis). *New Engl J Med.* 1988; 318: 684-688.
9. Evans DGR, Huson SM, Donnai D, et al. A clinical study of type 2 neurofibromatosis. *Q. J. Med.* 1992; 84: 603-618.

10. Evans DGR, Huson SM, Donnai D, et al. A genetic study of type 2 neurofibromatosis in the United Kingdom. I. Prevalence, mutation rate, fitness, and confirmation of maternal transmission effect on severity. *J Med Genet.* 1992; 29: 841-846.
11. Wishart JH. Case of tumors in the skull, dura mater, and brain. *Edinburgh Med Surg J.* 1822; 18: 393-397.
12. Gardner WJ, Frazier CH. Bilateral acoustic neurofibromatosis: A clinical study and field survey of a family of five generations with bilateral deafness in thirty-eight members. *Arch Neurol Psychiatry.* 1930; 23:266-302.
13. Ruggieri M, Huson SM. The clinical and diagnostic implications of mosaicism in neurofibromatosis. *Neurology.* 2001; 56(11): 1433-43.
14. Kluwe L, Mautner V, Heinrich B. Molecular study of frequency of mosaicism in neurofibromatosis 2 patients with bilateral vestibular schwannomas. *J Med Genet.* 2003; 40(2): 109-114.
15. Moyhuddin A, Baser MG, Watson C. Somatic mosaicism in neurofibromatosis 2: prevalence and risk of disease transmission to offspring. *J Med Genet.* 2003; 40(6): 459-63.
16. MacCollin M, Willett C, Heinrich B, et al. Familial schwannomatosis: Exclusion of the NF2 locus as the germline event. *Neurology.* 2003; 60(12): 1968-74.
17. Kameyama S, Tanaka R, Kawaguchi T, et al. Cystic acoustic neurinomas: studies of 14 cases. *Acta Neurochir.* 1996; 138: 695-699.
18. Charabi S, Tos M, Thomsen J, et al. Cystic vestibular schwannoma—clinical and experimental studies. *Acta Otolaryngol (Suppl).* 2000; 543: 11-13.
19. Charabi S, Tos M, Borgesen SE, et al. Cystic acoustic neuromas. results of translabyrinthine surgery. *Arch Otolaryngol Head Neck Surg.* 1994; 120: 1333-1338.
20. Fundova P, Charabi S, Tos M, et al. Cystic vestibular schwannoma: surgical outcome. *J Laryngol Otol.* 2000; 114: 935-39.
21. Pendl G, Ganz JC, Kitz K, et al. Acoustic neurinomas with macrocysts treated with Gamma Knife

- radiosurgery. *Stereotact Funct Neurosurg.* 1995; 66(Suppl. 1):103-111.
22. Shirato H, Sakamoto T, Takeichi N, et al. Fractionated stereotactic radiotherapy for vestibular schwannoma (VS): comparison between cystic-type and solid-type VS. *Int J Radiat Oncol Biol Phys.* 2000; 48: 1395-401.
23. Rouleau GA, Wertelecki W, Haines JL, et al. Genetic linkage of bilateral acoustic neurofibromatosis to a DNA marker on chromosome 22. *Nature.* 1987; 329(6136): 246-8.
24. Wertelecki W, Rouleau GA, Superneau DW, et al. Neurofibromatosis 2: Clinical and DNA linkage studies of a larger kindred. *New Engl J Med.* 1988; 319: 278-283.
25. Rouleau GA, Merel P, Lutchman M, et al. Alteration in a new gene encoding a putative membrane-organizing protein causes neurofibromatosis type 2. *Nature.* 1993; 363: 515-21.
26. Trofatter JA, MacCollin MM, Rutter JL, et al. A novel Moesin-, Exrin-, Radixin-like gene is a candidate for the neurofibromatosis 2 tumor-suppressor. *Cell.* 1993; 72: 791-800.
27. Welling DB, Guida M, Goll F, et al. Mutational spectrums in the neurofibromatosis type 2 gene in sporadic and familial schwannomas. *Hum Genet.* 1996; 98: 189-193.
28. Welling DB. Clinical manifestations of mutations in the neurofibromatosis type 2 gene in vestibular schwannomas (acoustic neuromas). *Laryngoscope.* 1998; 108: 178-189.
29. Jacoby LB, MacCollin M, Barone R, et al. Frequency and distribution of NF2 mutations in schwannomas. *Genes Chrom Cancer.* 1996; 17: 45-55.
30. Irving RM, Harada T, Moffat DA, et al. Somatic neurofibromatosis type 2 gene mutations and growth characteristics in vestibular schwannoma. *Am J Otol.* 1997; 18: 754-760.
31. Lekanne Deprez RH, Bianchi AB, Groen NA, et al. Frequent NF2 gene transcript mutations in sporadic meningiomas and vestibular schwannomas. *Genes Chrom Cancer.* 1994; 13: 211-216.
32. Merel P, Hoang-Xuan K, Sanson M, et al. Predominant occurrence of somatic mutations of the NF2 gene in meningiomas and schwannomas. *Genes Chrom Cancer.* 1995; 13: 1211-1216.
33. Merel P, Khe HX, Sanson M, et al. Screening for germ-line mutations in the NF2 gene. *Genes Chrom*

Cancer. 1995; 12: 117-127.

34. Lasota J, Fetsch JF, Wozniak A, et al. The neurofibromatosis type 2 gene is mutated in perineurial cell tumors: a molecular genetic study of eight cases. *Am J Pathol*. 2001; 158: 1223-1229.
35. Bianchi AB, Hara T, Ramesh V, et al. Mutations in transcript isoforms of the neurofibromatosis 2 gene in multiple human tumour types. *Nat Genet*. 1994; 6: 185-192.
36. Bianchi AB, Mitsunaga SI, Cheng JQ, et al. High frequency of inactivating mutations in the neurofibromatosis type 2 gene (NF2) in primary malignant mesotheliomas. *Proc Natl Acad Sci USA*. 1995; 92: 10854-10858.
37. Rutledge MH, Sarrazin J, Rangarathnam S, et al. Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. *Nat Genet*. 1994; 6: 180-184.
38. Sekido Y, Pass HI, Bader S, et al. Neurofibromatosis type 2 (NF2) gene is somatically mutated in mesothelioma but not in lung cancer. *Cancer Res*. 1995; 55: 1227-1231.
39. Deguen B, Goutebroze L, Giovannini M, et al. Heterogeneity of mesothelioma cell lines as defined by altered genomic structure and expression of the NF2 gene. *Int J Cancer*. 1998; 77: 554-560.
40. Sanson M, Marineau C, Desmaze C, et al. Germline detection in a neurofibromatosis type 2 kindred inactivates the NF2 gene and a candidate meningioma locus. *Hum Mol Genet*. 1993; 2: 1215-1220.
41. MacCollin M, Mohney T, Trofatter J, et al. DNA diagnosis of neurofibromatosis 2. *JAMA*. 1993; 270:2316-2320.
42. MacCollin M, Ramesh V, Jacoby LB, et al. Mutational analysis of patients with neurofibromatosis 2. *Am J Hum Genet*. 1994; 55: 314-320.
43. Deperez RHL, Bianchi AB, Groen NA, et al. Frequent NF2 Gene Transcript Mutations in Sporadic Meningiomas and Vestibular Schwannomas. *Am J Hum Genet*. 1994; 54: 1022-1029.
44. Irving RM, Moffat DA, Hardy DG, et al. Somatic NF2 gene mutations in familial and non-familial vestibular schwannoma. *Hum Mol Genet*. 1994; 3: 347-350.
45. Jacoby LB, MacCollin M, Louis DN, et al. Exon scanning for mutation of the NF2 gene in schwannomas.

Hum Mol Genet. 1994; 3 :413-419.

46. Jacoby LB, Jones D, Davis K, et al. Molecular analysis of the NF2 tumor-suppressor gene in schwannomatosis. Am J Hum Genet. 1997; 61: 1293-1302.
47. Sainz J, Figueroa K, Baser ME, et al. High frequency of nonsense mutations in the NF2 gene caused by C to T transitions in five CGA codons. Hum Genet. 1995; 4: 137-139.
48. Sainz J, Figueroa K, Baser ME, et al. Identification of three neurofibromatosis type 2 (NF2) gene mutations in vestibular schwannomas. Hum Mol Genet. 1996; 97: 121-123
49. Sainz J, Huynh DP, Figueroa K, et al. Mutations of the neurofibromatosis type 2 gene and lack of the gene product in vestibular schwannomas. Hum Mol Genet. 1994; 3: 885-891.
50. Twist EC, Ruttledge MH, Rousseau M, et al. The neurofibromatosis type 2 gene is inactivated in schwannomas. Hum Mol Genet. 1994; 3: 147-151.
51. Bourn D, Carter SA, Mason S, et al. Germline mutations in the neurofibromatosis type 2 tumour suppressor gene. Hum Mol Genet. 1994; 3: 813-816.
52. Bourn D, Evans G, Mason S, et al. Eleven novel mutations in the NF2 tumour suppressor gene. Hum Genet. 1995; 95: 572-574.
53. Kluwe L, Bayer S, Baser ME, et al. Identification of NF2 germ-line mutations and comparison with neurofibromatosis 2 phenotypes. Hum Genet. 1996; 98: 534-538.
54. Parry DM, MacCollin MM, Kaiser-Kupfer MI, et al. Germ-line mutations in the neurofibromatosis 2 gene:correlation with disease severity and retinal abnormalities. Am J Hum Genet. 1996; 59: 529-539.
55. Ruttledge MH, Andermann AA, Phelan CM, et al. Type of mutation in the neurofibromatosis type 2 gene (NF2) frequently determines severity of disease. Am J Hum Genet. 1996; 59: 331-342.
56. Gutmann DH, Geist RT, Xu Hm, et al. Defects in neurofibromatosis 2 protein function can arise at multiple levels. Hum Mol Genet. 1998; 7: 335-345.
57. Stokowski RP, Cox DR. Functional analysis of the neurofibromatosis type 2 protein by means of disease-causing point mutations. Am J Hum Genet. 2000; 66: 873-891.

58. Gutmann DH, Hirbe AC, Haipak CA. Functional analysis of neurofibromatosis 2 (NF2) missense mutations. *Hum. Mol. Genet.* 2001; 10: 1519-1529.
59. Bruder DEG, Kirvela C, Tapia-Paez I et al. High resolution deletion analysis of constitutional DNA from neurofibromatosis type 2 (NF2) patients using microarray-CGH. *Hum Molec Genet.* 2001; 10: 271-282.
60. Mautner VF, Baser ME, and Kluwe L. Phenotypic variability in two families with novel splice-site and frameshift NF2 mutations. *Hum. Genet.* 1996; 98: 203-206.
61. Zucman-Rossi J, Legoix P, Der Sarkissian H, et al. NF2 gene in neurofibromatosis type 2 patients. *Hum. Mol. Genet.* 1999; 7: 2095-2101.
62. Bruder CE, Ichimura K, Blennow E, et al. Severe phenotype of neurofibromatosis type 2 in a patient with a 7.4-MB constitutional deletion on chromosome 22: possible localization of a neurofibromatosis type 2 modifier gene? *Genes Chrom. Cancer.* 1999; 25: 184-190.
63. Kino T, Takeshima H, Nakao M, et al. Identification of the cis- acting region in the NF2 gene promoter as a potential target for mutation and methylation-dependent silencing in schwannoma. *Genes Cells.* 2001; 6(5): 441-54.
64. Chang LS, EM Akhmetiyeva, Y Wu, et al. Multiple transcription initiation sites, alternative splicing, and differential polyadenylation contribute to the complexity of human neurofibromatosis 2 transcripts. *Genomics.* 2002; 79: 63-76.
65. Algrain M, Arpin M, Louvard D. Wizardry at the cell cortex. *Current Biol.* 1993; 3: 451-54.
66. Chishti AH, Kim AC, Marfatia SM, et al The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *TIBS.* 1998; 23: 281-282.
67. Bretscher A, Chambers D, Nguyen R, et al. ERM-Merlin and EBP50 protein families in plasma membrane organization and function. *Annu. Rev. Cell. Dev. Biol.* 2000; 16: 113-43.
68. Turunen O, Wahlstrom T, Vaheri A. Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. *J. Cell Biol.* 1994; 126: 1445-1453.

69. Roy C, Martin M, Mangeat P. A dual involvement of the amino-terminal domain of ezrin in F- and G-actin binding. *J. Biol. Chem.* 1997; 272: 20088-20095.
70. Gonzalez-Agosti C, Xu L, Pinney D, et al. The merlin tumor suppressor localizes preferentially in membrane ruffles. *Oncogene*. 1996; 13: 1239-1247.
71. Pelton PD, Sherman LS, Rizvi TA, et al. Ruffling membrane, stress fiber, cell spreading and proliferation abnormalities in human schwannoma cells. *Oncogene*. 1998; 17: 2195-2209.
72. Deguen B, Merel P, Goutebroze L, et al. Impaired interaction of naturally occurring mutant NF2 protein with actin-based cytoskeleton and membrane. *Hum. Mol. Genet.* 1998; 7: 217-226.
73. Lutchman M, Rouleau GA. The neurofibromatosis type 2 gene product, schwannomin, suppresses growth of NIH 3T3 cells. *Cancer Res.* 1995; 55: 2270-2274.
74. Sherman L, Xu HM, Geist RT, et al. Interdomain binding mediates tumor growth suppression by the NF2 gene product. *Oncogene*. 1997; 15: 2505-2509.
75. Tikoo A, Varga M, Ramesh V, et al. An anti-Ras function of neurofibromatosis type 2 gene product (NF2/Merlin). *J. Biol. Chem.* 1994; 269: 23387-23390.
76. McClatchey AI, Saotome I, Mercer K, et al. Mice heterozygous for a mutation at the NF2 tumor suppressor locus develop a range of highly metastatic tumors. *Genes Dev.* 1998; 12: 1121-1133.
77. McClatchey AI, Saotome I, Ramesh V, et al. The NF2 tumor suppressor gene product is essential for extraembryonic development immediately prior to gastrulation. *Genes Dev.* 1997; 11: 1253-65.
78. Giovannini M, Robanus-Maandag E, van der Valk M, et al. Conditional biallelic *Nf2* mutation in the mouse promotes manifestations of human neurofibromatosis type 2. *Genes Dev.* 2000; 14: 1617-1630.
79. Shaw RJ, Paez JG, Curto M, et al. The NF2 tumor suppressor, merlin, functions in Rac-dependent signaling. *Dev. Cell.* 2001; 1: 63-72.
80. Fernandez-Valle C, Tang Y, Ricard J, et al. Paxillin binds schwannomin and regulates its density-dependent localization and effect on cell morphology. *Nat Genet.* 2000; 31(4): 354-62.
81. Gronholm M, Vossebein L, Carlson CR, et al. Merlin links to the cAMP neuronal signaling pathway by

- anchoring the R1 beta subunit of protein kinase A. *J Biol Chem.* 2003; 278(42): 41167-72.
82. Kissil JL, Wilker EW, Johnson KC, et al. Merlin, the product of the NF2 tumor suppressor gene, is an inhibitor of the p21-activated kinase, PAK1. *Mol Cell.* 2003; 12(4): 841-9.
83. Gonzalez-Agosti C, Wiederhold T, Herndon ME, et al. Interdomain interaction of merlin isoforms and its influence on intermolecular binding to NHE-RF. *J Biol Chem.* 1999; 274(48): 34438-42.
84. Takeshima H, Izawa I, Lee PS, et al. Detection of cellular proteins that interact with the NF2 tumor suppressor gene product. *Oncogene.* 1994; 9: 2135-2144.
85. Sainio M, Zhao F, Heiska L, et al. Neurofibromatosis 2 tumor suppressor protein colocalizes with ezrin and CD44 and associates with actin-containing cytoskeleton. *J. Cell Sci.* 1997; 110: 2249-60.
86. Huang L, Ichimaru E, Pestonjamasp K, et al. Merlin differ from moesin in binding to F-actin and in its intra- and intermolecular interactions. *Biochim. Biophys. Res. Comm.* 1998; 248: 548-553.
87. Murthy A, Gonzalez-Agosti C, Cordero E, et al. NHE-RF, a regulatory cofactor for Na⁽⁺⁾-H⁺ exchange, is a common interactor for merlin and ERM (MERM) proteins. *J. Biol. Chem.* 1998; 273: 1273-1276.
88. Obremski VJ, Hall AM, Fernandez-Valle C. Merlin, the neurofibromatosis type 2 gene product, and β1 integrin associate in isolated and differentiating Schwann cells. *J. Neurobiol.* 1998; 37: 487-501.
89. Scoles DR, Huynh DP, Morcos PA, et al. Neurofibromatosis 2 tumor suppressor schwannomin interacts with βII-spectrin. *Nat. Genet.* 1998; 18:354-359.
90. Scoles DR, Huynh DP, Chen MS, et al. The neurofibromatosis 2 tumor suppressor protein interacts with hepatocyte growth factor-regulated tyrosine kinase substrate. *Hum. Mol. Genet.* 2000; 9: 1567-1574.
91. Xu HM, Gutmann DH. Merlin differentially associates with the microtubule and actin cytoskeleton. *J. Neurosci. Res.* 1998; 51: 403-415.
92. Goutebroze L, Brault E, Muchardt C, et al. Cloning and characterization of SCHIP-1, a novel protein interacting specifically with spliced isoforms and naturally occurring mutant NF2 proteins. *Mol. Cell Biol.* 2000; 20: 1699-1712.
93. Herrlich P, Morrison H, Sleeman J, et al. CD44 acts both as a growth and invasiveness-promoting

- molecule and as a tumor-suppressing cofactor. Ann. NY Acad. Sci. 2000; 910: 106-18.
94. Sherman L, Sleeman J, Herrlich P, et al. Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. Curr. Opin. Cell Biol. 1994; 6: 726-33.
95. Morrison H, Sherman LS, Legg J, et al. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. Genes Dev. 2001; 15: 968-980.
96. Xiao GH, Beeser A, Chernoff J, et al. P21-activated Kinase links Rac/Cdc42 signaling to merlin. J Biol Chem. 2002; 277: 883-886.
97. Kissil JL, Johnson KC, Eckman MS, et al. Merlin phosphorylation by p21-activated kinase 2 and effects of phosphorylation on merlin localization. J Biol Chem. 2002; 277: 10394-9.
98. Bretscher A, Reczek D, Berryman M. Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. J. Cell Sci. 1997; 110: 3011-3018.
99. Tsukita S, Yonemura S, Tsukita S. ERM proteins: head-to-tail regulation of actin-plasma membrane interaction. Trends Biochem. Sci. 1997; 22: 53-58.
100. Hirao M, Sato N, Kondo T, et al. Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. J. Cell Biol. 1996; 135: 37-51.
101. Gutmann DH, Sherman L, Seftor L, et al. Increased expression of the NF2 tumor suppressor gene product, merlin, impairs cell motility, adhesion and spreading. Hum. Mol. Genet. 1999; 8: 267-275.
102. Gutmann DH, Haipek CA, Burke SP, et al. The *NF2* interactor, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), associates with merlin in the ‘open’ conformation and suppresses cell growth and motility. Hum Mol Genet. 2001; 10: 825-834.
103. Pearson MA, Reczek D, Bretscher A, et al. Structure of the ERM protein moesin reveals the FERM domain fold masked by an extended actin binding tail domain. Cell. 2000; 101: 259-270.
104. Komada M, Kitamura N. Growth factor-induced tyrosine phosphorylation of Hrs, a novel 115-kilodalton

- protein with a structurally conserved putative zinc finger domain. *Mol Cell Biol.* 1995; 15: 6213-6221.
105. Welling DB, Akhmadeteva EM, Daniels RL, et al. Analysis of the human neurofibromatosis type 2 gene promoter and its expression. *Otolaryngol Head Neck Surg.* 2000; 123: 413-418.
106. Arakawa H, Hayashi N, Nagase H, et al. Alternative splicing of the NF2 gene and its mutation analysis of breast and colorectal cancers. *Hum Mol Genet.* 1994; 3: 565-568.
107. Hitotsumatsu T, Kitamoto T, Iwaki T, et al. An exon 8-spliced out transcript of neurofibromatosis 2 gene is constitutively expressed in various human tissues. *J. Biochem.* 1994; 116: 1205-1207.
108. Koga H, Araki N, Takeshima H, et al. Impairment of cell adhesion by expression of the mutant neurofibromatosis type 2 (NF2) genes which lack exons in the ERM-homology domain. *Oncogene.* 1998; 17:801-810.
109. Nishi T, Takeshima H, Hamada K, et al. Neurofibromatosis 2 gene has novel alternative splicing which control intracellular protein binding. *Int J Oncol.* 1997; 10: 1025-1029.
110. Pykett MJ, Murphy M, Harnish PR, et al. The neurofibromatosis type 2 tumor suppressor gene encodes multiple alternatively spliced transcripts. *Hum Mol Genet.* 1994; 3: 559-564.
111. Schmucker B, Tang Y, Kressel M. Novel alternatively spliced isoforms of the neurofibromatosis type 2 tumor suppressor are targeted to the nucleus and cytoplasmic granules. *Hum Mol Genet.* 1999; 8: 1561-1570.
112. Giovannini M, Robanus-Maandag E, Niwa-Kawakita M, et al. Schwann cell hyperplasia and tumors in transgenic mice expressing a naturally occurring mutant NF2 protein. *Genes Dev.* 1999; 13: 978-986.
113. Labit-Bouvier C, Crebassa B, Bouvier C, et al. Clinicopathologic growth factors in vestibular schwannomas: a morphological and immunohistochemical study of 69 tumours. *Acta Otolaryngol.* 2000; 120: 950-4.
114. Niemczyk K, Vaneecloo FN, Lecomte MH, et al. Correlation between Ki-67 index and some clinical aspects of acoustic neuromas (vestibular schwannomas). *Otolaryngol Head Neck Surg.* 2000; 123: 779-83.

115. Light JP, Roland JT Jr, Fishman A, et al. Atypical and low-grade malignant vestibular schwannomas: Clinical implications of proliferative activity. *Otol Neurotol.* 2001; 22: 922-7.
116. Chen JM, Houle S, Ang LC, et al. A study of vestibular schwannomas using positron emission tomography and monoclonal antibody Ki-67. *Am J Otol.* 1998; 19: 840-5.
117. Kesterson L, Shelton C, Dressler L, et al. Clinical behavior of acoustic tumors. A flow cytometric analysis. *Arch Otolaryngol Head Neck Surg.* 1993; 119(3): 269-71.
118. Cardillo MR, Filipo R, Monini S, et al. Transforming growth factor-beta1 expression in human acoustic neuroma. *Am J Otol.* 1999; 20: 65-8.
119. Charabi S, Mantoni M, Tos M, et al. Cystic vestibular schwannomas: neuroimaging and growth rate. *J Laryngol Otol.* 1994; 108: 375-379.
120. Charabi S, Klinken L, Tos M, et al. Histopathology and growth pattern of cystic acoustic neuromas. *Laryngoscope.* 1994; 104: 1348-1352.
121. Antinheimo J, Haapasalo H, Seppala M, et al. Proliferative potential of sporadic and neurofibromatosis 2-associated schwannomas as studied by MIB-1 (Ki-67) and PCNA labeling. *J Neuropathol Exp Neurol.* 1995; 54(6): 776-82.
122. Aguiar PH, Tatagiba M, Samii M, et al. The comparison between the growth fraction of bilateral vestibular schwannomas in neurofibromatosis 2 (NF2) and unilateral vestibular schwannomas using the monoclonal antibody MIB1. *Acta Neurochir.* 1995; 134: 40-5.
123. Lander ES, Lenton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature.* 2001; 409(6822): 860-921.
124. Bassett DE Jr, Eisen MB, Boguski MS. Gene expression informatics -- it's all in your mind. *Nature Genet.* 1999; 21: 51-55.
125. Lockhart DJ, Winzeler EA. Genomics, gene expression, and DNA arrays. *Nature.* 2000; 405: 827-836.
126. DeRisi JL, Iyer VR, Brown PO. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science.* 1997; 278: 680-6.

127. Welling DB, JM Lasak, EM Akhdameteva, et al. cDNA microarray analysis of vestibular schwannomas. *Otol Neurotol.* 2002; 23(5): 736-48.
128. Lasak JM, DB Welling, M Salloum, et al. Deregulation of the pRb-CDK pathway in vestibular schwannomas. *Laryngoscope.* 2001; 112(9): 1555-61.
129. Zhang DH, Salto-Tellez M, Chiu LL, et al. Tissue microarray study for classification of breast tumors. *Life Sci.* 2003; 73(25): 3189-99.
130. Nishizuku S, Chen ST, Gwadry FG, et al. Diagnostic markers that distinguish colon and ovarian adenocarcinomas: identification, genomic, proteomic, and tissue array profiling. *Cancer Res.* 2003; 63(17): 5243-50.
131. Davis LD, Zhang W, Merseburger A, et al. p63 expression profile in normal and malignant epithelial cells. *Anticancer Res.* 2002; 22(6C): 3819-25.
132. Alizadeh AA, Ross DT, Perou CM, et al. Towards a novel classification of human malignancies based on gene expression patterns. *J Pathol.* 2001; 195(1): 41-52.
133. Jordan CT. Unique molecular and cellular features of acute myelogenous leukemia stem cells. *Leukemia.* 2002; 16:559-62.
134. Staudt LM. Gene expression profiling of lymphoid malignancies. *Annu Rev Med.* 2002; 53: 303-18.
135. Klausner, RD. Cancer, genomics, and the National Cancer Institute. *J Clin Invest.* 1999; 104: S15-7.
136. Vajkoczy P, Menger MD, Vollmar B, et al. Inhibition of tumor growth, angiogenesis, and microcirculation by the novel Flk-1 inhibitor SU-5416 as assessed by intravital multi-fluorescence videomicroscopy. *Neoplasia.* 1999; 1: 31-41.
137. Vajkoczy P, Menger MD, Goldbrunner R, et al. Targeting angiogenesis inhibits tumor infiltration and expression of the pro-invasive protein SPARC. *Int J Cancer.* 2000; 87: 261-8.
138. Allal C, Pradines A, Hamilton AD, et al. Farnesylation RhoB prevents cell cycle arrest and actin cytoskeleton disruption caused by the geranylgeranyltransferase I inhibitor GGTI-298. *Cell Cycle.* 2002; 1(6): 430-7.

FIGURE LEGENDS

Figure 1. MRI images of vestibular schwannomas. A) Axial T1 weighted MRI with gadolinium contrast. There is an enhancing right-sided cerebellopontine angle tumor with areas of central low intensity that correspond with cysts within this pathologically confirmed vestibular schwannoma. B) Axial T2 weighted MRI. The tumor is more hyperintense than the typical T2 signal characteristics of a vestibular schwannoma. Additionally, there are focal areas of increased signal intensity that correspond with the intra-tumoral cysts.

Figure 2: Schematic diagram of merlin action. This diagram shows how Rac1 and Pak help convert the merlin protein from a closed conformation to an open conformation by phosphorylation of the protein. Consequently, merlin, in its open conformation, can interact with CD44 and facilitate linking the actin cytoskeleton to the cell membrane.

Figure 3: The *NF2* gene is transcribed into mRNA; however, alternative splicing can produce different mRNA transcripts or isoforms. Different exon combinations can be brought together to produce multiple mRNAs from the same gene. Our studies showed that isoform I, II and delE15/16 were the most common isoforms found in vestibular schwannomas examined.

Figure 1

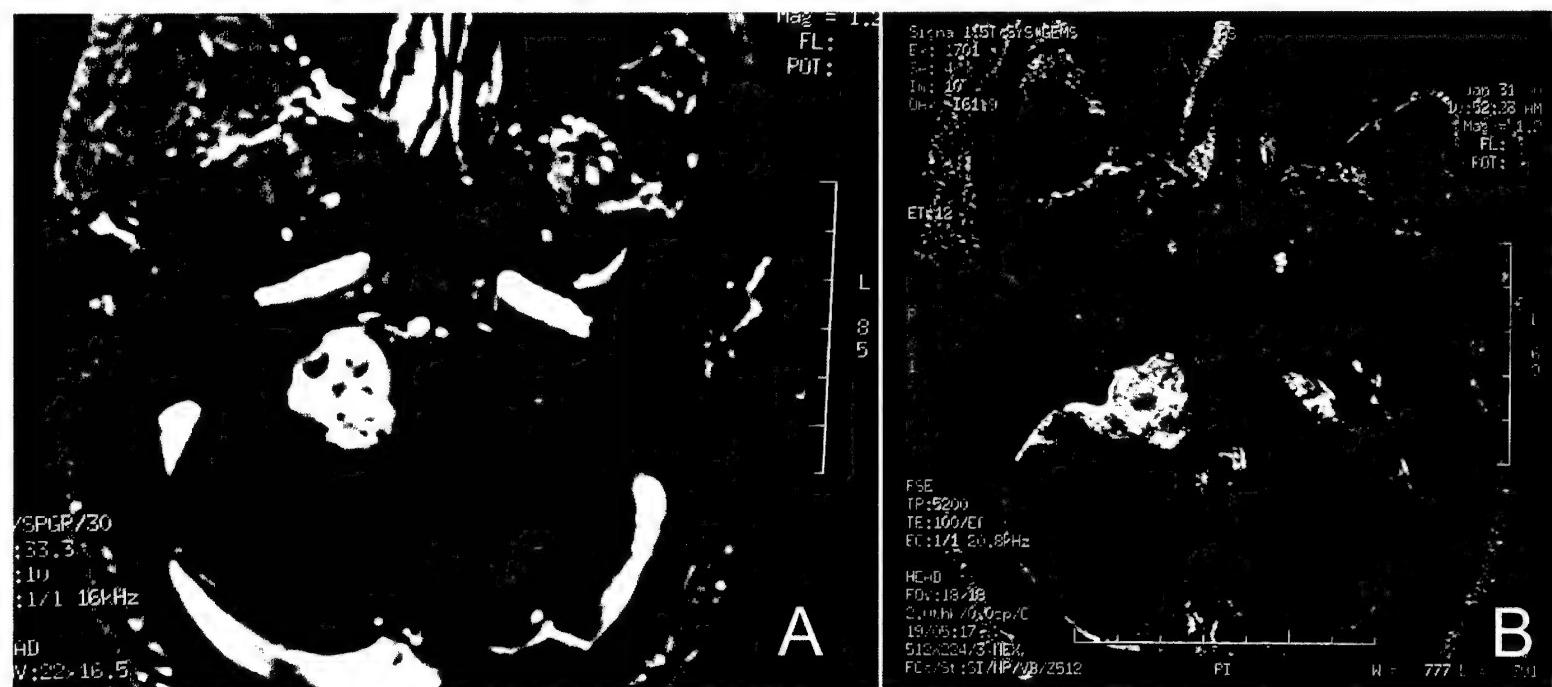
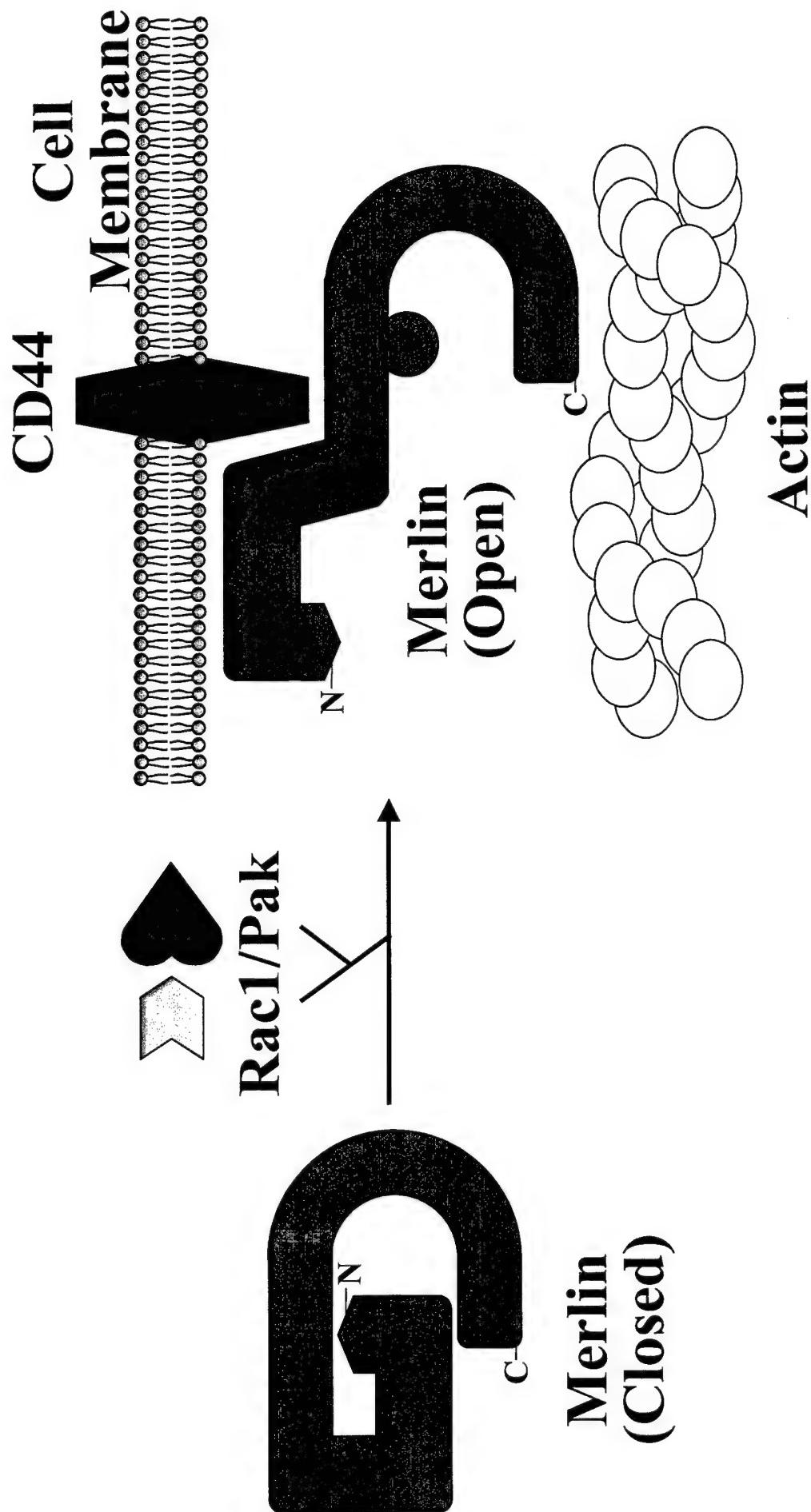


Figure 2



NF2 Isoform

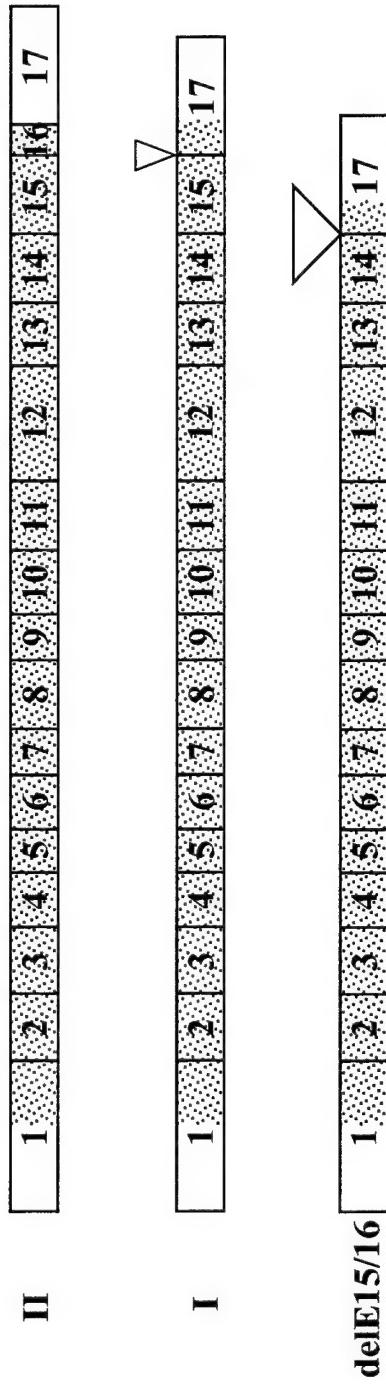


Figure 3

**VESTIBULAR SCHWANNOMAS XENOGRAFTED IN SCID MICE AND
EXPRESSION OF CYCLIN D1, D3, AND TELOMERASE**

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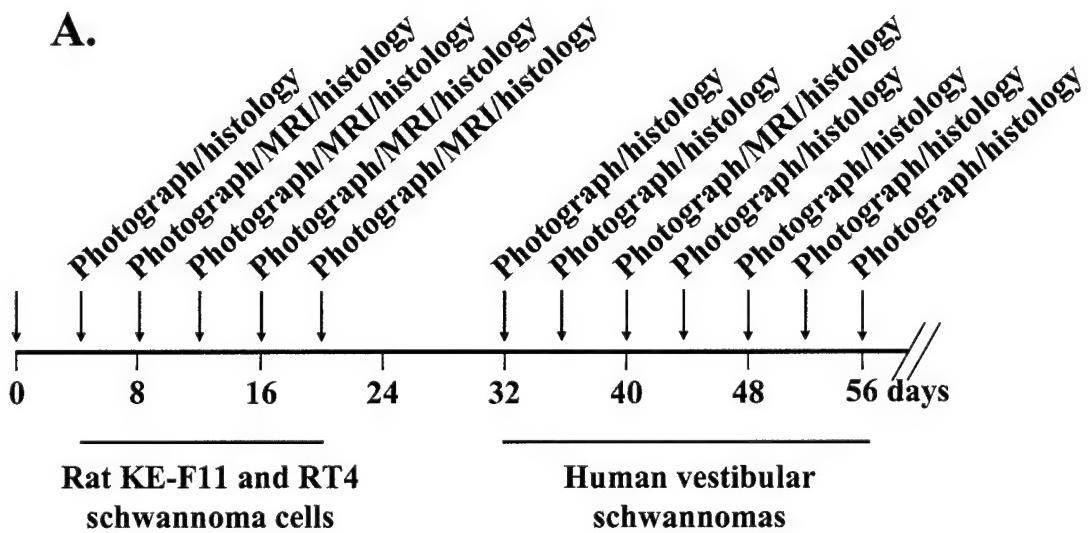
Abstract

Human vestibular schwannoma xenografts in immunocompromised mice was reported to be a useful model for the study of schwannoma biology. Although some tumor survival was observed, there was no long-term follow-up of tumor growth. Elegant works by McClatchey and colleagues, and by Giovaninni and colleagues showed that mice with a heterozygous or homozygous deletion of the *Nf2* gene displayed tumor formation. Intriguingly, none of these animal showed vestibular schwannoma formation. Thus, establishment of a human vestibular schwannoma xenograft model will be useful for further study leading to preclinical drug trials. To enhance tumor growth, 18 vestibular schwannoma tissues were minced and resuspended in Matrigel matrix, and then implanted into the sciatic nerve region subcutaneously in SCID mice. Two rat malignant schwannoma cell lines, RT-4 and KE-F11, were also used for comparison. Although minor growth of vestibular schwannoma implants was observed at the injection site initially, limited or no tumor growth was seen several (2~6) months after injection. MRI imaging of xenografted mice also revealed no tumor growth at the nerve or subcutaneous region. In contrast, visible tumors were seen in animals injected with both rat schwannoma cells (3×10^5 cells/mouse) and reached to the size larger than 0.5 cm in diameter two weeks after injection. It has been previously shown that cyclin D1 and D3 are important for Schwann cell proliferation and differentiation, respectively. To examine cyclin D1 and D3 expression, we performed immunostaining of human vestibular schwannoma tissue sections. Five of the ten vestibular schwannomas examined showed moderate staining of cyclin D3, while the rest showed little or no expression. Also, none of the 15 tumors examined showed cyclin D1 expression.

Human vestibular schwannoma cells have not been easily grown and senesce after limited passages in culture. Because the telomere is important for the life span of cells, we determined the length of telomere in schwannomas. When the rat RT-4 and KE-F11 schwannoma cell DNAs were digested with *Rsa*I and *Hinf*I enzymes, and probed with a telomere repeat sequence, hybridizing fragments of larger than 20 kb were detected. However, only moderate sizes (8~15 kb) of hybridizing telomere fragments were detected in human vestibular schwannoma DNAs. These results suggest multiple factors contribute to the growth potential of human vestibular schwannomas.

Results

Figure 1. The schwannoma xenograft model in SCID mice. (A) Timeline of the human vestibular schwannoma and rat malignant schwannoma xenograft experiment. (B) Photograph of a human vestibular schwannoma xenograft at day 40 after inoculation into the sciatic nerve region of a SCID mouse. (C) Photograph of the rat KE-F11 schwannoma xenograft at day 16 after inoculation. (D) Photograph of the rat RT-4 schwannoma xenograft at day 16 after inoculation. An arrow points to the region of tumor growth.



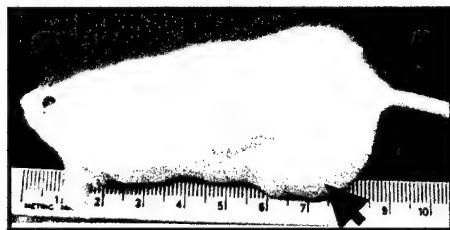
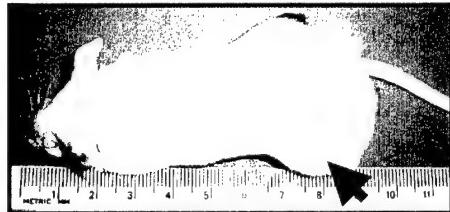
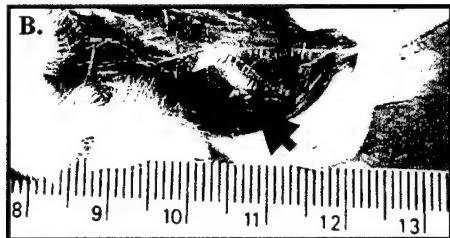


Figure 2. 4.7 Tesla MRI images of rat schwannoma xenografts in SCID mice

(A) KE-F11 schwannoma, RARE: 2500/54, RF8 200x200x800 μ m. (B) KE-11 schwannoma, SE: 620/10.2, Pre-Gd-DTPA. (C) KE-F11 schwannoma, SE: 620/10.2, Post-Gd-DTPA. (D) RT-4 schwannoma images, demonstrating cystic qualities.

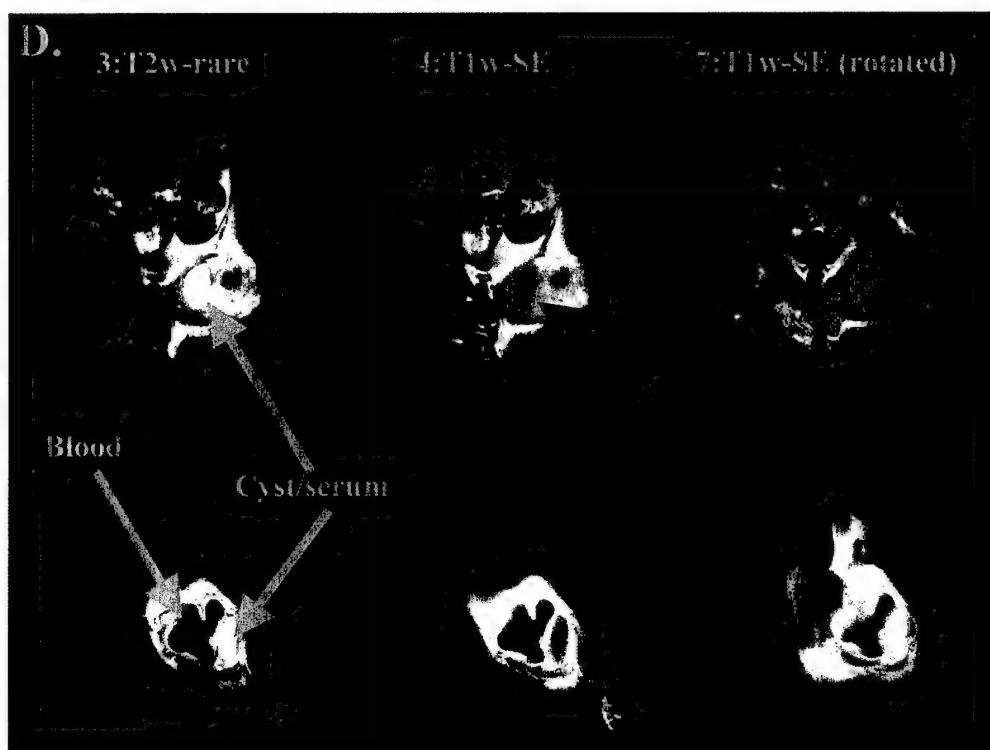


Fig. 3. Histology of xenograft tissue sections of (A) malignant rat KE-F11 and (B) RT-4 schwannomas. Sections of schwannoma xenografts at day 8 after inoculation were stained with hematoxylin and eosin. 40X magnification.

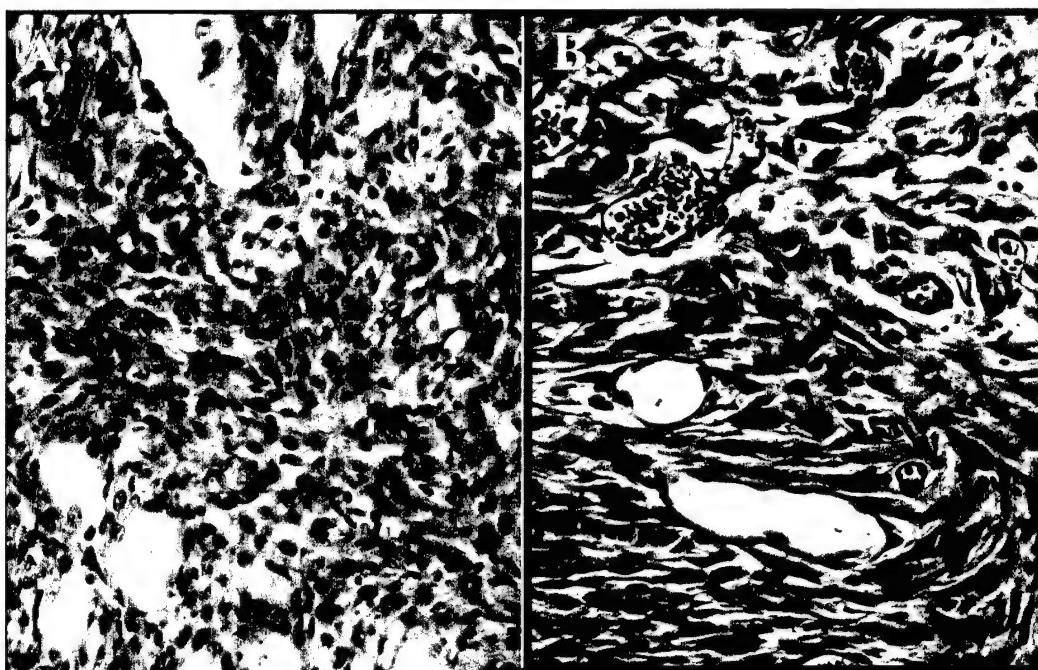


Table 1. Summary of Cyclin D₁ and D₃ immunostaining.

Sample No.	Cyclin D₁ Staining	Cyclin D₃ Staining
17796 A1	Negative	+ Nuclear Staining
17660 B1	Negative	Negative
1030012	Negative	
1030069	Negative	
0303C011	Negative	Negative
304577208	Negative	
17607	Negative	++ Nuclear Staining
17613	Negative	
17644	Negative	Negative
17638	Negative	++ Nuclear Staining
17579	Negative	
17666	Negative	+ Nuclear Staining
17661	Negative	+ Nuclear Staining
17789	Negative	Negative
17696	Negative	Negative

Fig. 4. Cyclin D₁ and D₃ immuno-reactivities in human vestibular schwannomas. As positive controls, sections of breast carcinoma (A) showing strong cyclin D₁ staining, and sections of prostate carcinoma (B) showing strong cyclin D₃ staining, were used. Rare Schwann cells (indicated by arrows) of the vestibular nerve (VN) (C) showed nuclear staining of cyclin D₃, but the staining was weaker than that in the positively stained vestibular schwannoma (D). Also, no cyclin D₁ staining was detected in the vestibular schwannoma tissue section (E).

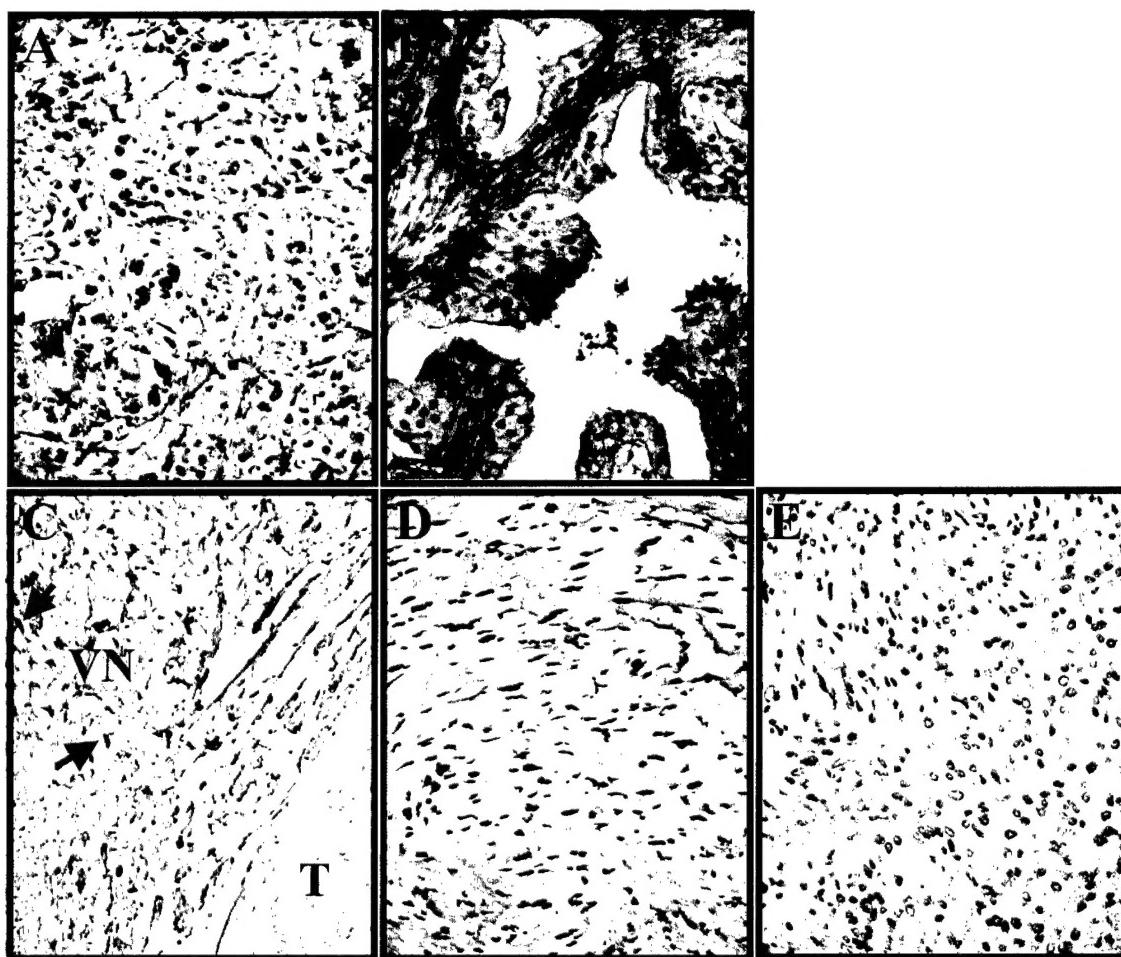
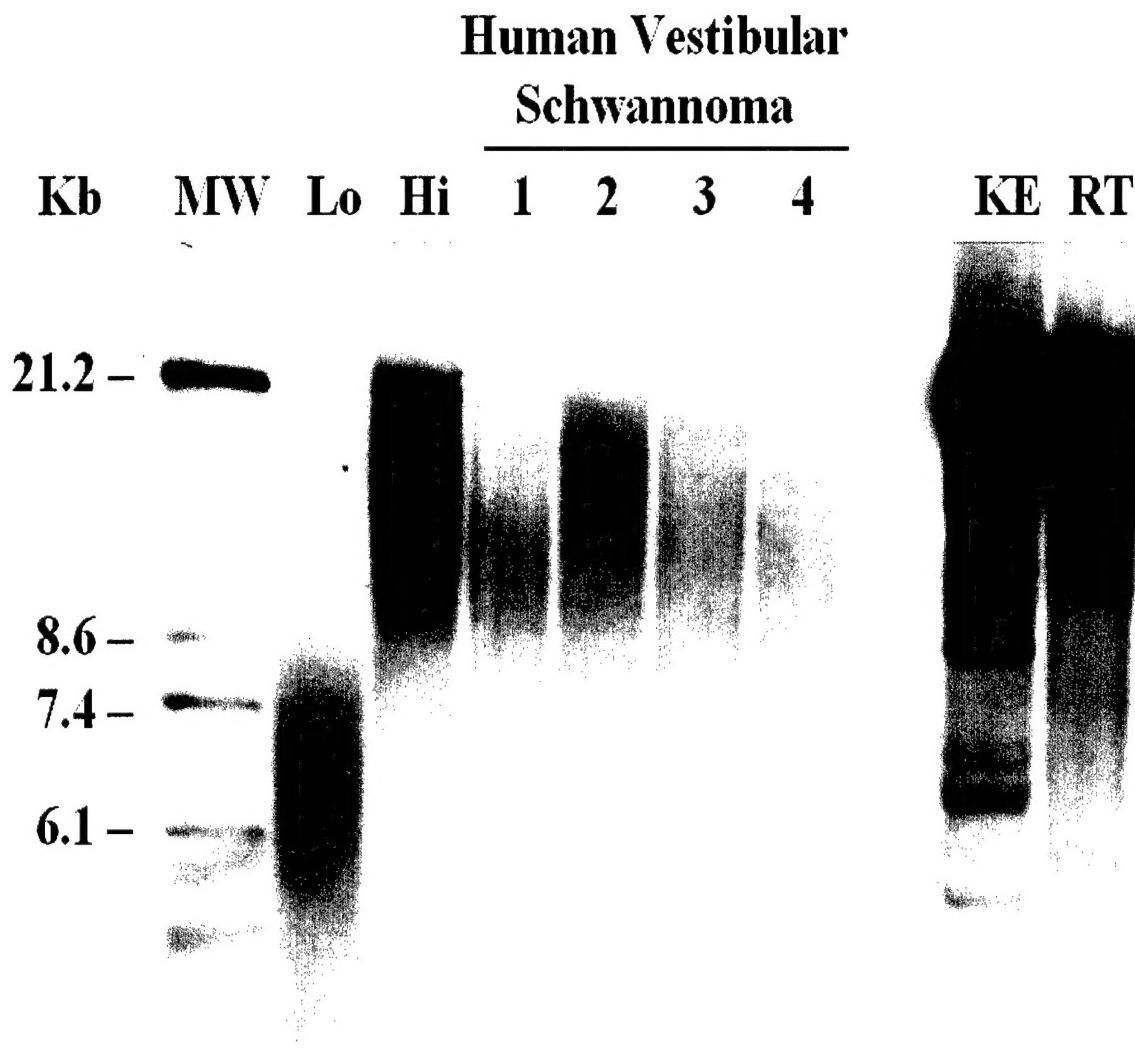


Fig. 5. Detection of telomere in vestibular schwannomas. Genomic DNAs isolated from human vestibular schwannomas and rat KE-F11 and RT-4 schwannomas (KE and RT) were digested with *Rsa*I and *Hinf*I enzymes, and probed with a telomere repeat sequence. Lo and Hi: telomere markers. MW: molecular weight marker.



Summary

1. Human vestibular schwannoma xenografts showed initial growth in SCID mice, but no long term tumor growth was observed *in vivo*.
2. In contrast, the RT-4 and KE-F11 rat schwannoma cells grew readily and produced large tumors in SCID mice within two weeks of inoculation.
3. MRI imaging demonstrates cystic tumors in RT-4 schwannoma xenografts and solid tumors in KE-F11 schwannoma xenografts.
4. Five of 10 vestibular schwannomas examined demonstrated cyclin D₃ expressions with scattered nuclear staining patterns; however, no cyclin D₁ expression was detected in 15 VS examined.
5. Human vestibular schwannomas contain moderate sized telomere fragments. In contrast, malignant rat schwannomas harbor long telomeres.

Conclusion

Our results suggest multiple factors contribute to the growth potential of human vestibular schwannomas. Schwannoma xenografts may be useful for future evaluation of therapeutic intervention.

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